THÈSE / UNIVERSITÉ DE BRETAGNE OCCIDENTALE
sous le sceau de l'Université européenne de Bretagne

pour obtenir le titre de
DOCTEUR DE L'UNIVERSITÉ DE BRETAGNE OCCIDENTALE
Mention : Biologie Marine
École Doctorale des Sciences de la Mer

présentée par

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Préparée à l'Institut Universitaire Européen de la Mer, au Laboratoire des Sciences de l'Environnement Marin (LEMAR)

Influence de facteurs biotiques et abiotiques sur la physiologie et la production d'acide domoïque de *Pseudo-nitzschia* spp.

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On ne va jamais si loin que lorsque l'on ne sait pas où l'on va.
Christophe Colomb

REMERCIEMENTS

Je voudrais tout d'abord adresser un grand merci à tous ceux qui ont participé, de près ou de loin, à ma thèse et mes études.

Un grand merci aux membres du jury, pour avoir rendu ce jour enfin possible: Dorothée Vincent, Pascal Claquin, Christian Jeanthon et mes chefs. Ainsi qu'aux rapporteurs Laure Guillou et Gary Wikfors.

Merci au LEMAR et à son directeur Laurent Memery, de m'avoir accueillie dans ses laboratoires et ses bureaux, si difficiles à obtenir.

Merci aussi et surtout à mes chefs, Philippe Soudant et Hélène Hégaret, pour leurs idées, leurs corrections plus ou moins rapides, leurs remarques, pour leur aide et leur soutien tout simplement. Je suis très fière d'avoir travaillé avec vous, je n'aurais pas pu mieux tomber.

Merci aussi à Eva Bucciarelli, qui a rendu tout ce travail sur les limitations en métaux possible. Je ne pensais pas que la chimie pouvait être si compliquée, et pourtant si simple quand tu l'expliques. A nos sessions skype, nos tentatives pour lier chimie et physiologie (et le pâté de crocodile).

Merci bien entendu à Nelly et Christophe, qui m'ont vu évoluer, et sans être directement et officiellement impliqués dans ma thèse, ont toujours su m'aider et me donner des idées quand il le fallait. Merci à Nelly de m'avoir toujours indiqué où chaque chose se cachait, comment chaque machine fonctionnait, sans jamais râler (ou si peu).

Merci à Dianne Jolley, pour cette rencontre du bout du monde et ces chocolats australiens.

A mes copines de galère, merci de votre soutien, de nos conversations déprimes, de filles ou de tout et de rien : Isabelle, Pauline, Maria et Célie, sans vous ca n'aurait pas été pareil.

Merci à mes voisins de bureau, qui se sont succédés mais ont tous rendu mes journées plus joyeuses, plus drôles, moins lassantes et parfois réussi l'exploit de me faire oublier mes soucis : Fanny, Leïla, Nolwenn, les 2 Franck, David, Morgana et Cédric.

Aux "nouveaux", c'est à votre tour, je vous souhaite plein de courage : Tony, JP, Romain, Séb, Julia (Cédric et Morgana aussi je vous souhaite du courage, surtout en étant dans le même bureau).

Aux "anciens" : Hansy, Leïla, MAT, Morgane, Sabine, Rossana, vous m'avez prouvé qu'avec un peu de courage et de détermination, il était possible de survivre à une thèse.

Merci à mes stagiaires, j'espère que vous n'êtes pas trop traumatisés et que vous avez trouvé votre voie : Mélanie Daoudal, Simon Bonato, Marc Long.

Un grand merci aussi à Geneviève Cohat et Anne-Sophie Podeur pour leur disponibilité et leur bonne humeur. Merci Gene pour les cours de danses bretonnes.

Merci également aux "chimistes", qui répondent présent quand il y a besoin : Marie Cheize, Manon Legoff, Géraldine Sarthou, Julia Boutorh, Aude Leynaert et j'en passe.

Sans oublier le reste du labo, Anne-Claire Baudoux, Marion Cardinaud, Véra Deschryver, Anne Donval, Caroline Fabioux, Morgane Galinari, Fabienne Guérard, Alain Lemercier, Alain Marhic, Annick Masson, Brivaëla Moriceau, Christine Paillard, Sabine Roussel, Valérie Stiger-Pouvreau (par ordre alphabétique pour ne pas faire de jaloux :p).

Gary, pour m'avoir accueillie, par deux fois en plus, aux USA.

Steve Bates, pour son aide précieuse à la rédaction de ma revue et ses connaissances sur *Pseudo-nitzschia*.

Aux personnes qui m'ont permis d'avoir des souches de *Pseudo-nitzschia* et autres sur lesquelles travailler : Catherine Dreanno, Beatriz Beker, Isabelle Quéau et Nancy Lewis.

A vous tous, j'espère que l'on se reverra un jour.

Je tiens à remercier du fond du cœur mes parents, ma famille et Franck, sans qui je ne serais jamais allée aussi loin. Merci de votre amour et de votre soutien, de vos sacrifices pendant toutes ces années d'étude.

SOMMAIRE

Introduction générale et objectifs ..... - 1 -

Chapitre 1 – Etat de l'art ..... - 7 -

Article 1 – *Pseudo-nitzschia* (Bacillariophyceae) species, domoic acid and amnesic shellfish poisoning: revisiting previous paradigms ..... - 9 -

Chapitre 2 – Comment suivre les variations physiologiques de *Pseudo-nitzschia* spp. ? ..... - 53 -

2.1. Préambule ..... - 55 -
2.2. Espèces modèles ..... - 56 -
2.3. Article 2 – Cell based measurements to assess physiological status of *Pseudo-nitzschia multiseries*, a toxic diatom ..... - 57 -
2.4. Conclusion ..... - 70 -

Chapitre 3 - Compétition entre *Pseudo-nitzschia multiseries* et une autre diatomée ..... - 71 -

3.1. Préambule ..... - 73 -
3.2. Article 3 - How does *Pseudo-nitzschia multiseries*, a toxic diatom, compete with diatoms of the *Chaetoceros* genus? ..... - 74 -
3.3. Conclusion ..... - 94 -

Chapitre 4 - Interactions entre *Pseudo-nitzschia* spp. et différentes communautés bactériennes... - 95 -

4.1. Préambule ..... - 97 -
4.2. Article 4 - Modification of domoic acid production and cell physiology after exchange of bacterial community between toxic *Pseudo-nitzschia multiseries* and non toxic *Pseudo-nitzschia delicatissima* ..... - 98 -
4.3. Conclusion ..... - 126 -

Chapitre 5 - Effets de doses toxiques de cuivre sur *Pseudo-nitzschia* spp. ..... - 127 -

5.1. Préambule ..... - 129 -
5.2. Article 5 – Impact of copper exposure on *Pseudo-nitzschia* spp. physiology and domoic acid production ..... - 130 -
5.4. Conclusion ..... - 141 -

Chapitre 6 - Effets de limitations en fer et/ou cuivre sur *Pseudo-nitzschia delicatissima*..... - 143 -

6.1. Préambule ..... - 145 -
6.2. Article 6 - Iron and copper limitations impact growth rates, photosynthetic and physiological parameters of the marine diatom *Pseudo-nitzschia delicatissima* ..... - 146 -
6.3. Conclusion ..... - 173 -

Synthèse, conclusions et perspectives ..... - 175 -

Bibliographie..... - 186 -

Annexes ..... - 194 -

Annexe 1 – Cytogrammes des communautés bactériennes ..... - 194 -
Annexe 2 - Article 7 – A new insight into allelopathic effects of *Alexandrium minutum* on photosynthesis and respiration of the diatom *Chaetoceros neogracile* revealed by photosynthetic-performance analysis and flow cytometry ..... - 199 -

Valorisation Scientifique..... - 212 -

INTRODUCTION GENERALE ET OBJECTIFS

Pour beaucoup, les "poumons" de la planète sont les forêts terrestres, et principalement la forêt d'Amazonie. Mais les plantes terrestres ne sont pas les seules, et encore moins les principales, à rendre l'atmosphère terrestre viable. En effet, le phytoplancton -qu'il soit marin, d'eau douce ou saumâtre- fournit entre 60 et 80 % de l'oxygène (O_2) atmosphérique et piège une grande partie du dioxyde de carbone (CO_2) atmosphérique. Sans phytoplancton océanique en effet, les concentrations atmosphériques de CO_2 augmenteraient de 150-200 ppmv, une fraction considérable comparée aux concentrations actuelles de 380 ppmv (Falkowski et al., 2000). Ce phytoplancton est composé de toutes les espèces unicellulaires capables de faire de la photosynthèse, qu'il s'agisse de microalgues ou de cyanobactéries (Larousse). Mais son rôle va bien au-delà de sa capacité à utiliser le CO_2 et libérer de l'O_2. Il est en effet à la base de la chaîne alimentaire aquatique. A l'heure actuelle, quelques milliers d'espèces seulement ont été décrites (Sournia et al., 1991). Sachant que de nouvelles espèces sont décrites chaque année, ce nombre a dû fortement augmenter et reste probablement très largement sous-estimé. Ces espèces de phytoplancton se divisent en 4 groupes principaux (les diatomées, les dinoflagellés, les coccolithophoridés et les cyanobactéries).

Certaines espèces de phytoplancton, dont des cyanobactéries, sont capables de former des efflorescences (Figure 1). Ces espèces se multiplient alors dans l'eau, jusqu'à atteindre des concentrations très importantes pouvant provoquer une coloration de l'eau (du vert aux différentes nuances de rouge/marron). En milieu marin, sur les ~ 6 000 espèces actuellement connues, environ 200 sont considérées comme toxiques (Smayda, 1997). Une espèce est dite toxique lorsqu'elle a des effets nocifs sur la santé humaine et/ou sur d'autres organismes, de façon directe ou indirecte. Le problème vient du fait que certaines de ces espèces toxiques sont également capables de former des efflorescences (par exemple Nézan et al., 2010). Ces espèces sont consommées par les maillons supérieurs de la chaîne alimentaire, les filtreurs, eux-mêmes consommés par des prédateurs, du poisson au mammifère marin en passant par les oiseaux et les êtres humains. Les toxines s'accumulent dans chacun des maillons de la chaîne alimentaire, entraînant des problèmes divers et variés, dépendant de la toxine et de sa quantité, et qui peuvent aller jusqu'à la mort de l'organisme intoxiqué (par exemple Pulido, 2008). Ainsi chaque année, des centaines, voire des milliers de poissons, mammifères marins

et oiseaux meurent empoisonnés (par exemple, plus de 400 lions de mer sont morts en Californie en 1998, Scholin et al., 2000).

Figure 1 : A. Efflorescence de coccolithophores, Bretagne, France, 15 juin 2004. B. Efflorescence de *Microcystis aeruginosa*, West Catawba Island, Lake Erie, USA, juillet 2009. *C.* Efflorescence de dinoflagellés à San Mateo, Californie, USA, 2009.

L'être humain étant potentiellement touché, des mesures de prévention existent dans de nombreux pays. La concentration des espèces toxiques et des toxines est régulièrement mesurée dans l'eau et/ou les bivalves commerciaux, de façon à en interdire la récolte et la commercialisation dès qu'il y a un risque potentiel (rôle de l'IFREMER et du réseau REPHY en France). Parmi les espèces étroitement surveillées, on retrouve les diatomées du genre *Pseudo-nitzschia*. Ce genre regroupe actuellement 37 espèces, réparties dans tous l'Océan mondial, dont une partie est capable de produire de l'acide domoïque (Lelong et al., 2012b). L'acide domoïque est une toxine dite amnésiante, homologue de l'acide glutamique, un neurotransmetteur, dont elle prend la place au niveau des synapses neuronales (Pulido, 2008). Cette fixation entraîne, entre autre, des pertes de mémoire à plus ou moins long terme puis la mort de l'organisme (Pulido, 2008). Les oiseaux, mammifères marins et êtres humains sont les plus touchés par ces symptômes. En France on retrouve le genre *Pseudo-nitzschia* très fréquemment à des concentrations allant de quelques centaines jusqu'à plusieurs dizaines de millions de cellules par litre (Figure 2).

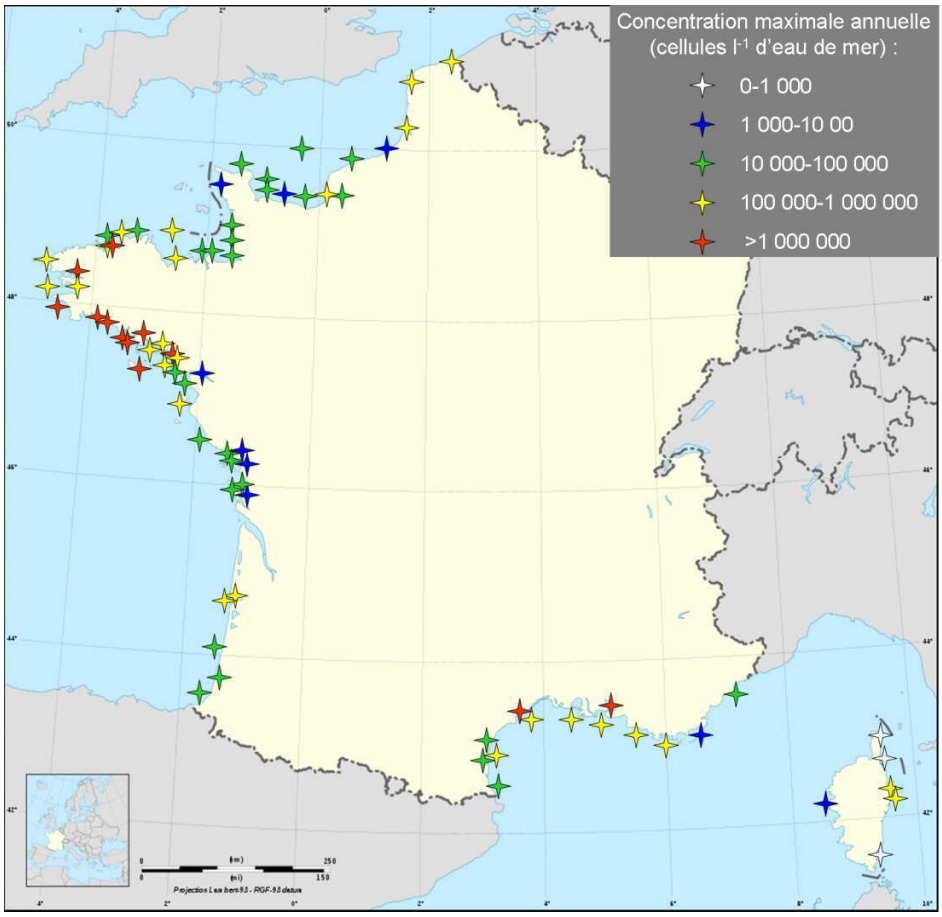

Figure 2 : Efflorescences de *Pseudo-nitzschia* spp. recensées en France pendant l'année 2008. D'après les données de l'IFREMER (http://envlit.ifremer.fr/var/envlit/storage/documents/parammaps/phytoplancton/index.html).

Le genre *Pseudo-nitzschia*, découvert en 1987 lors d'un évènement toxique ayant tué 3 personnes et rendu plus d'une centaine d'autres malades (Bates et al., 1989), a depuis été beaucoup étudié. Il s'est avéré que certaines espèces sont toxiques, d'autres pas, et que certaines espèces ont des souches toxiques et des souches non toxiques (Lelong et al., 2012b). Actuellement, aucune généralité n'a pu être établie pour savoir quand et pourquoi une souche est toxique ou non. Les diverses études se contredisent souvent, entre autre à cause de fortes différences entre espèces et même entre souches d'une même espèce. C'est pourquoi **le premier chapitre de cette thèse est un état de l'art**, aussi exhaustif que possible, qui regroupe les dernières avancées et découvertes faites sur le genre *Pseudo-nitzschia* (Lelong et al., 2012b)**.**

Nous avons ainsi décidé de nous intéresser, dans le cadre de cette thèse, à l'influence des facteurs biotiques (interaction avec les bactéries et d'autres espèces phytoplanctoniques) et abiotiques (impact des métaux fer et cuivre) sur la toxicité de deux espèces de *Pseudo-nitzschia*, *P. multiseries* et *P. delicatissima*. L'originalité de notre démarche réside dans notre

volonté de comprendre le lien entre toxicité et état physiologique. L'acide domoïque étant un composé secondaire, sa production n'est en effet pas essentielle à la croissance de la cellule. On peut donc supposer que cette production est liée à l'état physiologique des cellules et régulée pour une (des) raison(s) précise(s).

Pour confirmer cette hypothèse, il a tout d'abord fallu mettre au point des mesures de l'état physiologique de *Pseudo-nitzschia.* Le **deuxième chapitre** présente ainsi la mise au point des techniques pour la mesure de paramètres physiologique des cellules de *Pseudo-nitzschia* à l'aide de nouveaux outils (cytométrie en flux et "pulse-amplitude modulated" fluorimetry, ou PAM) (Lelong et al., 2012a), ainsi que les espèces sur lesquelles cette thèse a été menée. En utilisant ces techniques, les études présentées dans les chapitres 3 à 6 ont ensuite permis d'explorer le lien entre la production d'acide domoïque et un état physiologique précis, en conditions contrôlées de compétition biotique (bactéries et phytoplancton: chapitres 3 et 4) ou de limitation/stress par les métaux (fer et cuivre: chapitres 5 et 6).

Le troisième chapitre de cette thèse est centré sur la compétition entre deux diatomées et son impact sur la production d'acide domoïque, la physiologie de *Pseudo-nitzschia* et les communautés bactériennes associées à ces diatomées (Lelong et al., in prep.-a). Le genre *Pseudo-nitzschia* étant présent dans le monde entier, et capable de produire des efflorescences dans des conditions environnementales très diverses, il a nécessairement des mécanismes d'adaptation qui l'avantagent face à ses compétiteurs phytoplanctoniques, potentiellement via la production d'acide domoïque.

En l'absence de bactéries, une souche toxique ne produit plus, ou presque plus, d'acide domoïque (Bates et al., 1995). **Le quatrième chapitre** présente ainsi nos travaux pour étudier l'impact des bactéries sur la production d'acide domoïque (Lelong et al., in prep.-b). Nous avons ainsi voulu savoir si la présence de bactéries liées à une souche toxique pouvait rendre une souche, initialement non toxique, toxique. Et à l'inverse, les bactéries liées à une souche non toxique peuvent-elles faire perdre leur toxicité à une souche initialement toxique ? De plus, quel est le lien entre les bactéries, la production d'acide domoïque et l'état physiologique des cellules ?

Les travaux des deux chapitres suivants se focalisent sur l'hypothèse selon laquelle l'acide domoïque, un chélatant du fer et du cuivre, serait produit en conditions de toxicité par le cuivre et de limitation par le fer et le cuivre (Rue and Bruland, 2001, Maldonado et al., 2002, Wells et al., 2005). Pour étudier de façon plus directe ce lien entre l'acide domoïque et la disponibilité en fer et cuivre dans le milieu, deux conditions différentes ont été testées. **Le cinquième chapitre** s'intéresse à l'effet d'une dose toxique de cuivre. Deux espèces de *Pseudo-nitzschia*, une toxique et une non toxique, ont été utilisées afin de vérifier si la toxicité en cuivre peut induire la production d''acide domoïque même chez une espèce *a priori* non toxique, et ce en lien avec l'état physiologique des cellules (Lelong et al., 2012a). **Le sixième chapitre** s'est basé plus particulièrement sur l'hypothèse de Wells et al. (2005) supposant que chaque espèce de *Pseudo-nitzschia* peut être toxique et que cette toxicité est liée à la disponibilité en fer et cuivre, via la compétition pour ces micronutriments (Lelong et al., in prep.-c). Une espèce non toxique a donc été soumise à des limitations en fer et en cuivre afin de suivre la production potentielle d'acide domoïque. Le suivi de l'état physiologique des cellules a également permis de tester si la limitation en cuivre se résume, in fine, à une limitation en fer comme supposé par une partie de la communauté scientifique (par exemple Peers et al., 2005, Maldonado et al., 2006).

Enfin, la **dernière partie** de cette thèse est une synthèse des résultats obtenus dans les chapitres précédents.

ETAT DE L'ART 1

Article :

Lelong A., Hégaret H., Soudant P., Bates S. S. 2012. *Pseudo-nitzschia* (Bacillariophyceae) species, domoic acid and amnesic shellfish poisoning: revisiting previous paradigms. *Phycologia* 51(2):168-216.

Article 1 – Pseudo-nitzschia *(Bacillariophyceae) species, domoic acid and amnesic shellfish poisoning: revisiting previous paradigms*

Phycologia (2012) Volume 51 (2), 168–216 Published 12 March 2012

Pseudo-nitzschia (Bacillariophyceae) species, domoic acid and amnesic shellfish poisoning: revisiting previous paradigms

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LELONG A., HÉGARET H., SOUDANT P. AND BATES S.S. 2012. *Pseudo-nitzschia* (Bacillariophyceae) species, domoic acid and amnesic shellfish poisoning: revisiting previous paradigms. *Phycologia* 51: 168–216. DOI: 10.2216/11-37

Pseudo-nitzschia is a globally distributed diatom genus, some species of which produce domoic acid (DA), the neurotoxin that causes amnesic shellfish poisoning. This toxin killed at least three humans in 1987, launching numerous studies concerning the identification, distribution, ecology and physiology of *Pseudo-nitzschia* spp. Since previous reviews in 1998, knowledge has been gained about the fate of DA, including its accumulation by marine animals and its degradation by light and bacteria. Molecular techniques and more precise microscopy have enabled the description of new *Pseudo-nitzschia* species, 15 since 2002, including ones that are cryptic and pseudo-cryptic. An increasing number of the 37 identified species, including oceanic and coastal species, have been studied in laboratory culture. The sexual reproduction of 14 species has been documented. Fourteen species have now been shown to be toxigenic, although some strains are not always toxic under the testing conditions. The biotic and abiotic factors that modify DA production are reviewed, with a focus on how new discoveries have changed our original hypotheses about control mechanisms. Recent studies confirm that silicate and phosphate limitation trigger DA production. However, stress by low concentrations of iron or high concentrations of copper are newly discovered triggers, suggesting a trace-metal chelation role for DA. Organic sources of nitrogen (urea and glutamine), as well as changes in pH, CO_2, salinity and bacterial concentration, also enhance DA production. Laboratory and field studies sometimes give divergent results for conditions that are conducive to toxin production. Gaps in knowledge include further information about the whole genome of *Pseudo-nitzschia* (including sexual stages), mechanisms of DA production and decline, presence or absence of a resting stage, heterotrophic ability, impact of viruses and fungi, and a more complete description of the ecological and physiological roles of DA.

CONTENTS

Introduction 169
Domoic acid 169
General characteristics 169
Removal and degradation 171
Degradation/stability under different conditions 171
Bacterial degradation 172
Photodegradation 172
Pseudo-nitzschia spp. 172
General characteristics 172
Identification 174
Reproduction 180
Producers of DA 182
Blooms 183
Physical parameters affecting growth and toxicity . . .186
Growth phase 186
Temperature 187
Salinity 189
Irradiance 190
pH 191
Nutritional parameters affecting growth and toxicity . . 192
Macronutrients 192
Silicon and phosphorus 192
Nitrogen 192
Organic nutrients and heterotrophic ability . . 193
Carbon 193
Trace metal nutrients 194
Lithium 194
Iron and copper 194
Biological parameters affecting growth and toxicity . . 196
Association with other phytoplankton 196
Allelopathy 197
Bacteria 197
Viruses and fungi 198
Impacts of domoic acid 198
Gaps in knowledge 202
Physiological and ecological roles of DA production 202
Presence/absence of a resting stage 202
Genomics of sexual stages 202
Whole genome of *Pseudo-nitzschia* spp. and complete DA biosynthetic pathway 202
Mechanisms of DA production and decline . . .203
Biological control of DA production and of bloom dynamics 203
Heterotrophic ability 203
Resolution of conflicting findings 203
Ability to predict blooms reliably 203
Summary 204
Acknowledgements 204
References 204

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INTRODUCTION

Diatoms of the *Pseudo-nitzschia* genus (Heterokonta, Bacillariophyceae) were first described by H. Peragallo (1897–1908), before Hustedt included them in the *Nitzschia* genus in 1958. Hasle (1994) then redescribed *Nitzschia* as a specific genus: *Pseudo-nitzschia* (summarized by Bates 2000). Contrary to *Nitzschia*, the long and narrow *Pseudo-nitzschia* cells form chains ('stepped colonies') by slightly overlapping their cell tips (Figs 1–3), among other small differences (Hasle 1994).

Interest in the *Pseudo-nitzschia* genus increased after 1987, when the first amnesic shellfish poisoning (ASP) event occurred, caused by human consumption of blue mussels (*Mytilus edulis*) containing the neurotoxin domoic acid (DA) (Bates *et al.* 1989). This event resulted in at least three deaths of elderly people and over 100 illnesses (reviewed by Bates *et al.* 1998; Pulido 2008; Trainer *et al.* 2008). The toxin was traced to a bloom of *Pseudo-nitzschia multiseries*, upon which the mussels had been feeding. This was the first time that a diatom was shown to produce a neurotoxin. Until this event DA was not considered as a toxin, although it was known as an anthelmintic treatment used by Japanese to rid young children of intestinal worms (Wright *et al.* 1989). However, an order-of-magnitude-lower concentration was used compared with the amount that the affected adults had consumed during the toxic mussel event.

Interest further increased when DA caused the death of seabirds (Fritz *et al.* 1992; Work *et al.* 1993) and marine mammals (Scholin *et al.* 2000), thus the alternative name domoic acid poisoning (DAP), and after it was found to be transferred up the food web by various vectors (see below, and summarized by Bargu *et al.* in press). Fortunately, no known human deaths have occurred since the original 1987 mussel poisoning incident, although consumers in France were affected when they ate uninspected shellfish (*Donax trunculus*) in 2000 (Thébaud *et al.* 2005). This is because surveillance programs worldwide now monitor the concentration of *Pseudo-nitzschia* species in seawater or the presence of DA in the flesh of molluscan shellfish and finfish destined for human consumption (Anderson *et al.* 2001). The harvesting and sale of seafood products is prohibited when the internationally accepted regulatory limit of 20 μg DA g^{-1} wet weight of tissue is attained (Wekell *et al.* 1994). These monitoring programs have taken advantage of research findings, since 1987, that have shed some light on which *Pseudo-nitzschia* species produce DA, the physical factors that may control bloom dynamics and location, and the environmental factors that are conducive for toxin production.

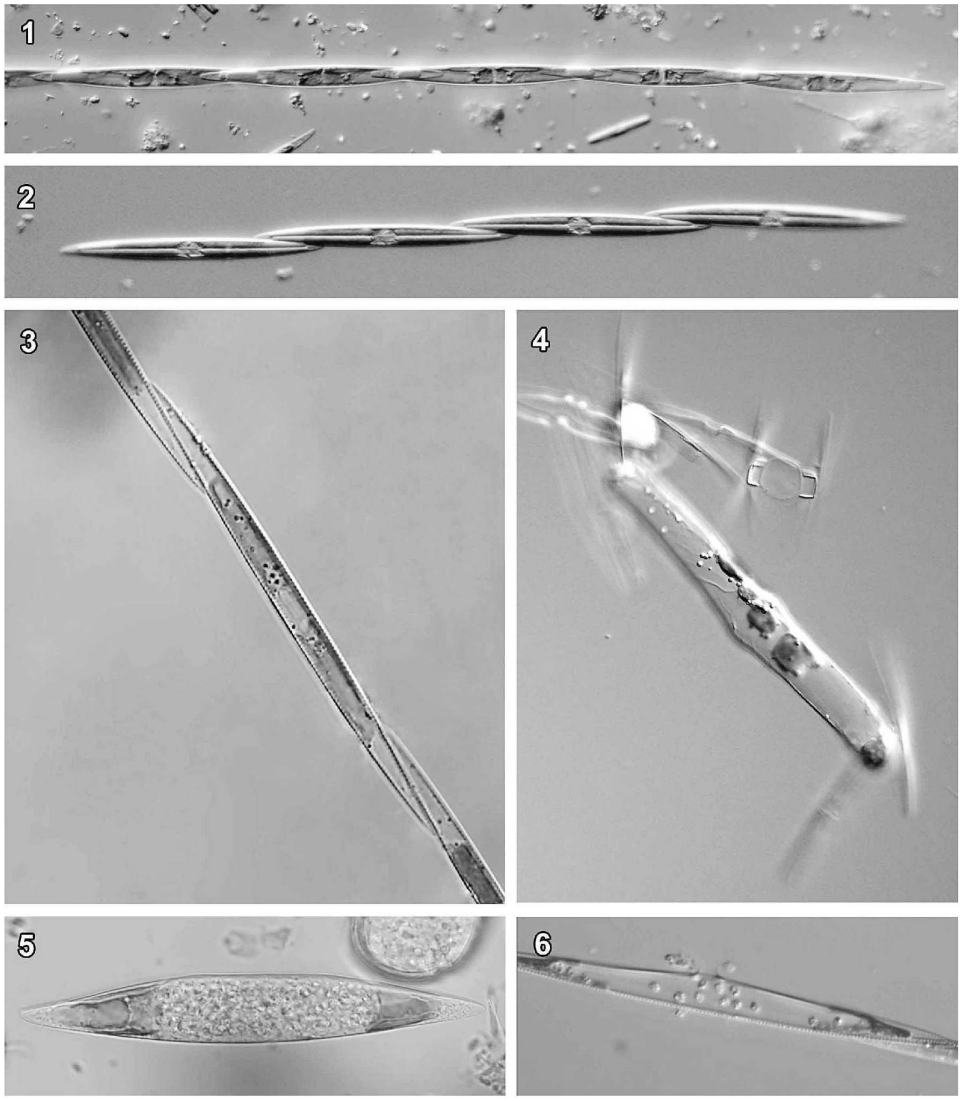
The goal of this review is to highlight some of the more recent important research advances regarding *Pseudo-nitzschia* biology and DA production in the context of previous paradigms. Updates since Bates (1998) and Bates *et al.* (1998) will be given on the chemistry of DA, fate of DA, new toxigenic species of *Pseudo-nitzschia*, distribution of these species and their blooms, biology of *Pseudo-nitzschia*, environmental factors conducive for *Pseudo-nitzschia* growth, and biotic and abiotic factors that trigger DA production. Other reviews have covered the occurrence of *Pseudo-nitzschia* species on the US west coast up to 1996 (Fryxell *et al.* 1997), the physiological ecology of *Pseudo-nitzschia* species up to 1998 (Bates 1998; Bates *et al.* 1998), general aspects (Todd 1993; Mos 2001; Jeffery *et al.* 2004), the ecology of this diatom genus (Bates & Trainer 2006), the molecular basis of DA toxicity (Ramsdell 2007), toxicologic pathology (Pulido 2008), *Pseudo-nitzschia* biology, including oceanographic factors that lead to toxic blooms (Trainer *et al.* 2008), risks of DA to wildlife (Bejarano *et al.* 2008b), molecular approaches for identifying *Pseudo-nitzschia* spp. and assessing its physiology (Kudela *et al.* 2010), the neurotoxicity of DA (Costa *et al.* 2010), vectors of DAP (Bargu *et al.* 2011a, b) and human exposure risks (Grant *et al.* 2010; Lefebvre & Robertson 2010). Additional literature is found in an updated, searchable compilation of DA and *Pseudo-nitzschia* references (Bates 2011).

DOMOIC ACID

General characteristics

Domoic acid is a water-soluble (Falk *et al.* 1991) amino acid of 311 Da, containing three carboxyl groups, and is an analogue of L-glutamic acid, a neurotransmitter, and of kainic acid (Falk *et al.* 1989). It has several geometrical isomers (isodomoic acids A, B, C, D, E, F, G and H) and the diastereoisomer epidomoic acid (Fig. 7) (de la Iglesia *et al.* 2008). Some of these are produced by *Chondria armata* (Maeda *et al.* 1986; Zaman *et al.* 1997), *Nitzschia navis-varingica* (Kotaki *et al.* 2005; Romero *et al.* 2011), *P. australis* (Holland *et al.* 2005; Rhodes *et al.* 2006) and *P. seriata* (Hansen *et al.* 2011). Others are found in molluscan shellfish, sometimes as degradation products (Wright *et al.* 1990; Vale & Sampayo 2001; Holland *et al.* 2003; Rhodes *et al.* 2004; Costa *et al.* 2005b; Holland *et al.* 2005), including photodegradation (see below). Epi-DA is a product of heat degradation (Thomas *et al.* 2008; McCarron *et al.* 2011). Isodomoic acids C (Clayden *et al.* 2005), G and H (Ni *et al.* 2009; Denmark *et al.* 2011), and B, E and F (Lemière *et al.* 2011) have been synthesized; DA has not yet been synthesized.

DA biosynthesis requires high levels of ATP (Pan *et al.* 1996a) and its pathway in *Pseudo-nitzschia* has only partially been resolved (Douglas *et al.* 1992; Thessen 2007). After ingestion, it can bind to *N*-methyl-D-aspartate receptors in the central nervous system with a coefficient 3 times greater than that of kainic acid and 100 times greater than that of glutamic acid (Teitelbaum *et al.* 1990). Because DA is not released by the neuron, in contrast to glutamic acid, depolarization is longer than it should be, thus increasing intraneuronal calcium concentration. Calcium-dependent enzyme activity is maintained, resulting in neuron swelling and then death. Neurons situated in the hippocampus (where memories are consolidated) are affected in mammals (Pulido 2008), leading to anterograde amnesia or short-term memory loss, hence the name ASP. The intoxication is followed by diverse symptoms, from gastric (nausea, diarrhea, gastroenteritis, cramps) and confusion in the first 24 h, to neurological (ataxia, headaches, breathing difficulties, disorientation, dizziness, memory loss) and sometimes coma in the 48 h after



Figs 1–6. *Pseudo-nitzschia* species. Photo credit: Karie Holtermann and E. Virginia Armbrust (University of Washington, Seattle, WA, USA).

Fig. 1. Valve view of a *P. australis* chain, showing cells attached by their overlapping apices, sampled during a toxic bloom (Cabrillo Beach, CA, USA; 09 March 2011). Note that the chain is slightly curved.

Fig. 2. Girdle view of a *P. multiseries* chain in culture; the cells are in the initial stages of dividing (strain originated from Goleta Beach, CA, USA; 10 February 2011).

Fig. 3. Close-up of a *P. multiseries* cell in a chain (Goleta Beach, CA, USA; 10 February 2011).

Fig. 4. Auxospore of *P. australis*; observed after crossing two cultures containing cells of opposite mating type. Note empty parental frustules on upper left of auxospore; the frustules are short as a result of continuous vegetative division and slightly deformed, an artifact sometimes seen after prolonged growth in culture; differential interference microscopy (DIC).

Fig. 5. Girdle view, showing a swollen, fungal-infected *P. pungens* cell containing oomycete zoospores (Goleta Beach, CA, USA; 10 February 2011); DIC.

Fig. 6. A fungal-infected *P. pungens* cell, showing discharging zoospores (Hood Canal, WA, USA; March–April 2007); DIC.

intoxication (Teitelbaum *et al.* 1990). Death may then occur. There is still no antidote to ASP.

Symptoms depend on the quantity of DA ingested and on the health of the affected person. Elderly people and those with impaired renal function or a compromised blood–brain barrier are the most sensitive to DA intoxication. DA is more toxic when consumed with the shellfish than is pure DA (Novelli *et al.* 1992) because of DA

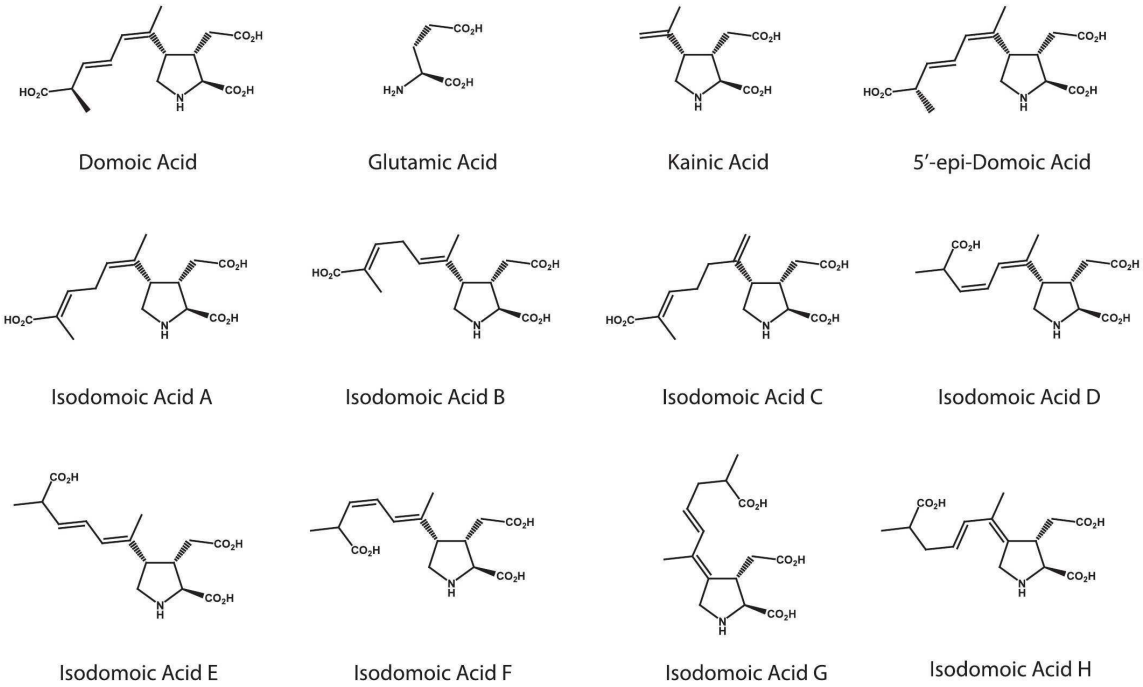


Fig. 7. Molecular structure of domoic acid and its isomers, glutamic acid and kainic acid (Courtesy of M. Quilliam, NRCC).

potentiation, caused by the high concentrations of glutamic and aspartic acids in shellfish tissues. DA isomers bind less strongly to kainate receptors and are therefore less toxic than DA, with iso-F 2.7-fold less potent, iso-C, -D and -E 23 to 29-fold less potent and iso-B more than 95-fold less potent than DA (Sawant *et al.* 2007, 2010; Munday *et al.* 2008). Munday *et al.* (2008) and Sawant *et al.* (2007) found that iso-A was less toxic than DA, whereas Sawant *et al.* (2010) reported that iso-A is not significantly different from DA; however, different techniques were used.

Removal and degradation

DA does not accumulate in the water column because the low quantities produced are diluted into the vast oceans or sink to the depths while still within intact *Pseudo-nitzschia* cells (Sekula-Wood *et al.* 2009, 2011; Silver *et al.* 2010). Less than 20% of the available DA was adsorbed onto humic acids in the colloidal phase, and adsorption onto natural seawater particles and suspended sediment was negligible (< 5%), but there were no losses onto suspensions of clay minerals (Lail *et al.* 2007); isomeric DA behaved similarly. Bacterial and photodegradation are the more important pathways for its elimination (see below).

DEGRADATION/STABILITY UNDER DIFFERENT CONDITIONS: Interest in the stability of DA stems from the desire to: (1) prevent DA degradation during transport and storage of shellfish samples prior to DA measurement in biotoxin monitoring programs; (2) develop efficient methods to extract DA from toxic cells and shellfish tissues; and (3) prevent DA degradation in certified reference materials (CRMs) of aqueous solutions and tissue samples used to calibrate instruments.

Stability studies showed that DA plus epi-DA extracted from king scallops (*Pecten maximus*) into aqueous methanol degraded by ~ 15% and ~ 45% in the whole-animal extracts and gonad extracts, respectively, over a 2-wk period (Smith *et al.* 2006). Indeed, methanol is known to degrade DA (Vale & Sampayo 2001). Extracts should not be stored in methanol but rather in citric acid buffer, which was shown to be stable over a 3-mo period.

For DA extraction, exposure time to high temperature and the extraction solvent used are critical in determining the stability of DA. The original Association of Official Analytical Chemists-approved method for DA determination used the paralytic shellfish poisoning toxin acid extraction method, i.e. homogenizing the sample for 5 min in boiling 0.1 N HCl. However, this acidic medium (but not the boiling) results in a 5–10% loss in DA, so it has been replaced by an aqueous methanolic (1:1) extraction solution (summarized in Quilliam 2003). Regarding temperature stability, boiling Dungeness crabs (*Cancer magister*) in fresh or salt water for 20 min did not degrade the DA, although its concentration was reduced by 67–71% because the hydrophilic toxin leached from the shellfish tissues into the surrounding water (Hatfield *et al.* 1995). Boiling whole cultures of *Pseudo-nitzschia* (cells plus medium) in test tubes for 3 min efficiently extracted DA from toxic diatom cells, with no significant loss of toxin compared with the control (Bajarias *et al.* 2006). Such extracted material was stable at

Table 1. Continued

Species	Group (cell width)	Toxicity	References for toxicity	Geographical distribution	References for distribution
P. multistriata (Takano) Takano	neither (2.5–3.7)	yes/no	Rhodes *et al.* 2000	Australia, Brazil, China, France (Atlantic), Greece, Gulf of Mexico, Gulf of Naples, Japan, Korea, New Zealand, Portugal, Russia (NW Sea of Japan, Sea of Okhotsk), Spain (Atlantic, Mediterranean), Thailand, Uruguay, Vietnam	Takano 1995; Hasle 2002; Orsini *et al.* 2002; Skov *et al.* 2004; Lundholm *et al.* 2003; Quijano-Scheggia *et al.* 2005, 2008; Thessen *et al.* 2005; Churro *et al.* 2009; D'Alelio *et al.* 2009; Méndez & Ferrario 2009; Moschandreou *et al.* 2010; Orive *et al.* 2010; Stonik *et al.* 2011
P. obtusa (Hasle) Hasle & Lundholm	*seriata* (2.9–5.0)	no		Alaska, Barents Sea, Canadian Arctic, Denmark, Greenland, Gulf of St. Lawrence, Hudson Strait, Ireland, Newfoundland, Norway, Russia (Sea of Okhotsk, Kamchatka)	Hasle 2002; Hasle & Lundholm 2005; Stonik *et al.* 2011
P. prolongatoides (Hasle) Hasle	*delicatissima* (0.5–2.5)	not tested		Gulf of Mexico, Weddell Sea (Antarctica)	Almandoz *et al.* 2008; Krayevsky *et al.* 2009
P. pseudodelicatissima[3] (Hasle) Hasle	*delicatissima* (1.1–2.1)	yes/no	Lundholm *et al.* 1997	Australia, Bay of Fundy, Chile, China, Croatia, Denmark, France (Atlantic), Greece, Gulf of Maine, Gulf of Mexico, Gulf of Naples, Iceland, Ireland, Mexico (Gulf of Mexico), New Zealand, Norway, Portugal, Russia (Black Sea, Baltic Sea), Russian Arctic seas (White Sea), Scotland, Spain (Atlantic Mediterranean), Thailand, US west coast, Vietnam	Martin *et al.* 1990; Bates *et al.* 1993a; Hasle *et al.* 1996; Fraga *et al.* 1998; Rhodes 1998; Pan *et al.* 2001; Hasle 2002; Lundholm *et al.* 2003; Kaczmarska *et al.* 2005b; Churro *et al.* 2009; Moschandreou *et al.* 2010; Orive *et al.* 2010; Ljubešić *et al.* 2011
P. pungens (Grunow ex Cleve) Hasle	*seriata* (2.2–5.4)	yes/no	Rhodes *et al.* 1996	Argentina, Australia, Bay of Fundy, Bering Sea, Brazil, British Columbia, Caribbean Sea, Chesapeake Bay, Chile, China, Denmark, Ecuador, France (Atlantic, Mediterranean), Greece, Gulf of Mexico, Gulf of St. Lawrence, Hong Kong, Indian Ocean, Indonesia, Ireland, Japan, Korea, Mexico (Pacific, Gulf of Mexico), Morocco, Mozambique, New Zealand, Norway, Peru, Portugal, Russia (NW Sea of Japan, Sea of Okhotsk, Bering Sea, Black Sea, Baltic Sea), Russian Arctic seas (White Sea), Scotland, Spain (Atlantic, Mediterranean), Thailand, US east coast, US west coast, Vietnam, West Africa	Forbes & Denman 1991; Bates *et al.* 1993a, 1998; Hallegraeff 1994; Hasle *et al.* 1996; Rhodes *et al.* 1998; Hasle 2002; Lundholm *et al.* 2003; Kaczmarska *et al.* 2005b; Procopiak *et al.* 2006; Almandoz *et al.* 2007; Thessen & Stoecker 2008; Moschandreou *et al.* 2010; Orive *et al.* 2010; Stonik *et al.* 2011
P. pungiformis (Hasle) Hasle	*seriata* (4.0–5.0)	not tested		Monterey Bay	Hasle *et al.* 1996; Fryxell *et al.* 1997
P. roundii Hernández-Becerril	*seriata* (4.6–6.5)	not tested		Mexico (Pacific)	Hernández-Becerril & Díaz-Almeyda 2006

Table 1. Continued

Species	Group (cell width)	Toxicity	References for toxicity	Geographical distribution	References for distribution
P. seriata (Cleve) H. Peragallo	*seriata* (4.6–8.0)	yes/no	Lundholm *et al.* 1994	Argentina (Beagle Channel), Baltic Sea, Barents Sea, Bay of Fundy, Canadian Arctic (Resolute Bay), Chesapeake Bay, Denmark, Greece, Greenland, Gulf of St. Lawrence, Iceland, Ireland, Newfoundland, North Sea, Norway, Russia (NW Sea of Japan, Sea of Okhotsk), Russian Arctic seas (White Sea, Barents Sea), Scotland, US northeast coast	Bates *et al.* 1998; Lundholm *et al.* 1994; Hasle *et al.* 1996; Hasle 2002; Lundholm *et al.* 2003; Fehling *et al.* 2004; Kaczmarska *et al.* 2005b; Davidson & Fehling 2006; Procopiak *et al.* 2006; Almandoz *et al.* 2009; Ignatiades & Gotsis-Skretas 2010; Stonik *et al.* 2011; Hansen *et al.* 2011
P. sinica Qi, Ju & Lei	neither (2.5–5.0)	not tested		China, Thailand, Vietnam	Qi *et al.* 1994; Priisholm *et al.* 2002; Li *et al.* 2005; Doan-Nhu *et al.* 2008
P. subcurvata (Hasle) Fryxell	*delicatissima* (1.5–2.0)	no		Argentina, Drake Passage (Antarctica), Gulf of Mexico, Weddell Sea (Antarctica)	Fryxell *et al.* 1991; Bates *et al.* 1993a; Almandoz *et al.* 2007, 2008; Aké-Castillo & Okolodkov 2009
P. subfraudulenta (Hasle) Hasle	*seriata* (5.0–7.0)	no		Australia, Chile, China, France (Mediterranean), Greece, Gulf of Mexico, Gulf of Naples, Gulf of Panama, Japan, Korea, Mexico (Pacific, Gulf of Mexico), Monterey Bay, Northwest Africa, Vietnam	Hasle 1965, 2002; Skov *et al.* 2004; Thessen *et al.* 2005; Hernández-Becerril & Díaz-Almeyda 2006; Álvarez *et al.* 2009; Moschandreou *et al.* 2010
P. subpacifica (Hasle) Hasle	*seriata* (5.0–7.0)	no		Australia, Bay of Fundy, California, Chesapeake Bay, China, France (Atlantic), Gulf of Mexico; Gulf of Panama, Ireland, Korea, Monterey Bay, Northwest Africa, Portugal, Spain (Atlantic), Washington	Hasle 1965; Hallegraeff 1994; Fryxell *et al.* 1997; Fraga *et al.* 1998; Cho *et al.* 2001; Lundholm *et al.* 2003; Kaczmarska *et al.* 2005b; Thessen & Stoecker 2008; Churro *et al.* 2009; Orive *et al.* 2010
P. turgidula (Hasle) Hasle	neither (2.5–5.0)	yes	Rhodes *et al.* 1996	Argentina, Australia, Barents Sea, Bay of Fundy, China, Drake Passage, Monterey Bay, New Zealand, Scotland, Subarctic Pacific, Weddell Sea (Antarctica)	Hallegraeff 1994; Rhodes *et al.* 1996; Hasle 2002; Ferrario *et al.* 2004; Almandoz *et al.* 2007, 2008; Leandro *et al.* 2010a; Trick *et al.* 2010
P. turgiduloides Hasle	*delicatissima* (1.7–2.5)	no		Argentina, Drake Passage, Ross Sea, Weddell Sea (Antarctica)	Lundholm *et al.* 2003; Ferrario *et al.* 2004; Almandoz *et al.* 2007, 2008

[1] Some species may be *P. arenysensis* (*sensu* Quijano-Scheggia *et al.* 2009).
[2] Shipboard batch culture of natural seawater containing *P. granii* as the only species of *Pseudo-nitzschia*.
[3] May be multiple species within the *P. pseudodelicatissima* complex (*sensu* Lundholm *et al.* 2003; Amato & Montresor 2008), if reports are earlier than 2003.

molecular studies, as well as with mating studies, especially because of the number of cryptic and pseudo-cryptic species being identified (see below). As well, growth temperature has been shown to affect cell morphology, e.g. for *P. multiseries* (Lewis *et al.* 1993) and *P. seriata* (Lundholm *et al.* 1994; Fehling *et al.* 2004; Hansen *et al.* 2011), further confusing species identification. We must still not lose sight of the importance of training qualified taxonomists to work alongside those using molecular approaches.

Molecular studies involve sequence analyses of the nuclear-encoded large-subunit (LSU) ribosomal DNA (rDNA) (Stehr *et al.* 2002; Amato *et al.* 2007; McDonald *et al.* 2007; Thessen *et al.* 2009; Moschandreou *et al.* 2010) or of the internal transcribed spacer (ITS1 or ITS2) regions (e.g. Lundholm *et al.* 2003, 2006; Orsini *et al.* 2004; Amato *et al.* 2007; Kaczmarska *et al.* 2008; Casteleyn *et al.* 2009b; Thessen *et al.* 2009; Andree *et al.* 2011), the mitochondrion-encoded cytochrome *c* oxidase 1 (Kaczmarska *et al.* 2008; Lundholm *et al.* in press) or the chloroplast-encoded large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbc*L) (Amato *et al.* 2007; Casteleyn *et al.* 2009b, 2010; Lundholm *et al.* in press) or small (*rbc*S) (Delaney *et al.* in press) gene. Such molecular information has been used to develop an automated ribosomal intergenic spacer analysis approach to identify *Pseudo-nitzschia* species rapidly in environmental samples (Hubbard *et al.* 2008; Marchetti *et al.*

2008). McDonald *et al.* (2007) identified different species and pseudo-cryptic species in the natural environment by amplifying LSU fragments, which appeared to be more reliable than microscopic observations. Microsatellite markers, requiring isolates in culture, have been developed for *P. pungens* (Evans & Hays 2004), *P. multiseries* (Evans *et al.* 2004), *P. multistriata* (Tesson *et al.* 2011) and *P. australis* (N. Adams, personal comunication). These can be used to help determine if *Pseudo-nitzschia* populations are made up of cryptic species, different varieties of the same species or contain hybrid forms of species varieties (reviewed in Trainer *et al.* in press).

Phylogenetic trees on the basis of LSU rDNA sequence analysis have proven that *Pseudo-nitzschia* is a paraphyletic genus (Lundholm *et al.* 2002b). Often, the above groupings on the basis of cell width are consistent with those shown in phylogenetic trees (e.g. Cerino *et al.* 2005). The taxonomic status and phylogeny of *P. americana*, however, is still not clear because it is uncertain if the original sequences submitted came from the single-celled or chain-forming strains (Lundholm *et al.* 2002a; Orsini *et al.* 2002).

On the basis of molecular evidence and careful light microscopy examination of existing species, some of these species have recently been emended and several new ones defined (included in Table 1). It is likely that new species will continue to be discovered in this way, thus increasing the known species diversity (Medlin & Kooistra 2010; Lundholm *et al.* in press). *Pseudo-nitzschia pseudodelicatissima* was split into three species: *P. calliantha*, *P. pseudodelicatissima* and *P. caciantha*, and *P. cuspidata* was redefined (Lundholm *et al.* 2003). The genotype identified as *P. calliantha*2 (Amato *et al.* 2007) was named as a new species, *P. mannii*, within the *P. pseudodelicatissima/cuspidata* complex (Amato & Montresor 2008). Furthermore, a recent more thorough examination of the above narrow (~ 1.5–2.5 μm) species has revealed even greater diversity, with the identification of two new species: *P. hasleana* and *P. fryxelliana* (Lundholm *et al.* in press). Reports of DA production by strains identified as *P. pseudodelicatissima* before 2003 should therefore be questioned. *Pseudo-nitzschia seriata* f. *obtusa* was raised in rank to *P. obtusa* (Hasle & Lundholm 2005). *Pseudo-nitzschia delicatissima* was redefined and split into two new species: *P. dolorosa* and *P. decipiens* (Lundholm *et al.* 2006). Later, the *P. delicatissima* del1 strain of Amato *et al.* (2007) was named a new species, *P. arenysensis* (Quijano-Scheggia *et al.* 2009b). The detection of three ITS1 fragments, which differed from any known species, were assumed to correspond to uncultivated, and therefore not yet discovered, *Pseudo-nitzschia* species (Hubbard *et al.* 2008).

The molecular approach has also allowed the discovery of cryptic species, i.e. those that are morphologically identical, or too similar to be distinguished, but genetically different, so they are reproductively isolated from other members of their own 'species'. For example, Amato *et al.* (2007) showed that *P. pseudodelicatissima* is in fact a group of five species (*P. cuspidata*, *P. calliantha*, *P. calliantha*2, *P. caciantha* and *P. pseudodelicatissima*) and that *P. delicatissima* is a group of three species (*P. delicatissima*, *P. delicatissima*2 and *P. dolorosa*). Pseudo-cryptic species are likewise genetically distinct but also have minor ultrastructural differences that are difficult to detect (Amato *et al.* 2005). McDonald *et al.* (2007) found three new genotypes among the *Pseudo-nitzschia* by studying subtle morphological differences and LSU rDNA sequences: two were from within the *galaxiae* clade and one was possibly of an undescribed *delicatissima*-like cell. Such studies document the process of speciation and may also eventually help to explain the existence of toxic and nontoxic strains of presumably the same species.

Using both nuclear-encoded rDNA ITS and plastid-encoded *rbc*L sequences, three distinct clades (I–III) of *P. pungens* have been revealed that are also distinguishable by subtle differences in frustule ultrastructure (Casteleyn *et al.* 2010). The clades differ in their geographical distribution: clade I (*P. pungens* var. *pungens*) has a cosmopolitan distribution in temperate waters of the Atlantic and Pacific oceans; clade II (*P. pungens* var. *cingulata*), originally described by Villac & Fryxell (1998), has only been found in the northeastern Pacific, where it co-occurs with clade I; and clade III (*P. pungens* var. *aveirensis*), described by Churro *et al.* (2009), occurs in the tropical to warm-temperate waters of the Atlantic and Pacific oceans. Clades I and II are sexually compatible in culture (Casteleyn *et al.* 2008), and hybrids have been found in the field (Casteleyn *et al.* 2009a; Holtermann *et al.* 2010). So far, the correlation between the reported abilities of some strains of *P. pungens* to produce small quantities of DA (e.g. < 0.2 pg DA cell^{-1}) (see Bates *et al.* 1998; Calu *et al.* 2009) and the clade to which they belong has not been investigated. These studies support the existence of factors that limit gene flow in a presumably cosmopolitan species, and how this can lead to speciation.

Reproduction

Pseudo-nitzschia spp. exhibit asexual and sexual reproduction. In asexual reproduction, the parental hypotheca (the smaller theca) becomes the epitheca (the larger theca) of the daughter cell. A new hypotheca will then be formed within that smaller epitheca. Thus, the cells become smaller each time they divide. Because of the rigid frustule, sexual reproduction is generally required to restore the largest cell size of *Pseudo-nitzschia* spp. (Fig. 10). Unusually, Pan *et al.* (2001) observed a vegetative cell enlargement of *P.* cf. *pseudodelicatissima* on five occasions in monocultures. Sexual reproduction (also called auxosporulation) occurs when daughter cells reach a lower size threshold (Davidovich & Bates 1998; Hiltz *et al.* 2000; Amato *et al.* 2005) as a result of continued vegetative cell division (except for the unusual abrupt reduction in cell length; Chepurnov *et al.* 2005). This threshold, however, is not universal and is species dependent, often having a wide size range, e.g. for *P. delicatissima*, 20–90% of the maximal cell length (Amato *et al.* 2005); for *P. multiseries*, 23–70% (Hiltz *et al.* 2000; Bates & Davidovich 2002); 20–60% for *P. pungens* (Chepurnov *et al.* 2005); and 20–90% for *P. delicatissima* (Amato *et al.* 2005). In a field study, the upper size thresholds for sexual induction were ~ 62% and 75% of the maximum size for *P. pungens* and *P. australis*, respectively (Holtermann *et al.* 2010). Below a given size window, cells can no longer reproduce sexually and will eventually die.

To date, sexual reproduction has been reported, in laboratory cultures, for 14 *Pseudo-nitzschia* species and one

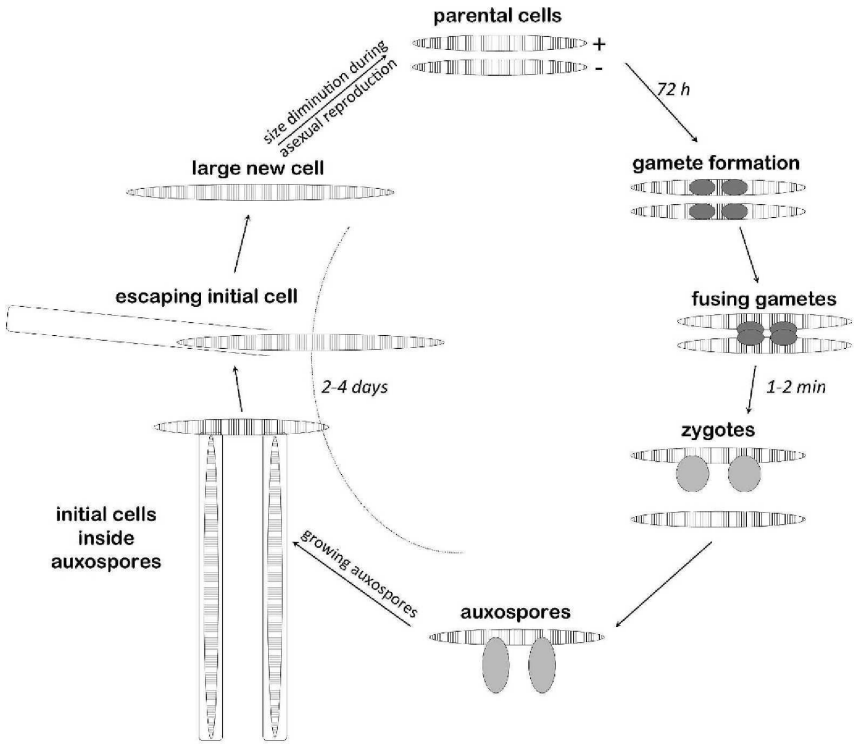


Fig. 10. Sexual reproduction of *Pseudo-nitzschia* sp., carried out to restore the large cell size, after size diminution as a result of asexual reproduction (vegetative cell division).

variety: *P. arenysensis* (Quijano-Scheggia *et al.* 2009b), *P. australis* (Holtermann *et al.* 2010), *P. brasiliana* (Quijano-Scheggia *et al.* 2009a), *P. calliantha* (Amato *et al.* 2007; Lundholm *et al.* in press), *P. cuspidata* (Lundholm *et al.* in press), *P. delicatissima* (Amato *et al.* 2005, 2007; Kaczmarska *et al.* 2008; Quijano-Scheggia *et al.* 2009b), *P. dolorosa* (Amato *et al.* 2007), *P. fraudulenta* (Chepurnov *et al.* 2004), *P. mannii* (Amato & Montresor 2008), *P. multiseries* (Davidovich & Bates 1998; Hiltz *et al.* 2000; Kaczmarska *et al.* 2000), *P. multistriata* (D'Alelio *et al.* 2009), *P. pseudodelicatissima* (Davidovich & Bates 1998; which may be *P. cuspidata*; Lundholm *et al.* 2003), *P. pseudodelicatissima sensu stricto* (Amato *et al.* 2007), *P. pungens* (Chepurnov *et al.* 2004, 2005; Casteleyn *et al.* 2008), *P. pungens* var. *aveirensis* (Churro *et al.* 2009) and *P. subcurvata* (Fryxell *et al.* 1991). Mating compatibility studies are increasingly being used to confirm species differences (Davidovich & Bates 1998; Amato *et al.* 2007; Casteleyn *et al.* 2008; Orlova *et al.* 2008; Quijano-Scheggia *et al.* 2009b; Lundholm *et al.* in press).

Most *Pseudo-nitzschia* species are heterothallic, requiring two parental cells (gametangia) of opposite mating type, a '+' and a '−' (Chepurnov *et al.* 2005; D'Alelio *et al.* 2009). Exceptions are *P. brasiliana*, which is homothallic, i.e. it does not require the opposite mating type (Quijano-Scheggia *et al.* 2009a), and *P. subcurvata*, which produced auxospores in a clonal culture and is therefore also apparently homothallic (Fryxell *et al.* 1991). After several hours, the + and − gametangia find each other and align themselves in a parallel fashion, without mucilage or any physical link. Sexual reproduction may occur between two single cells or between a cell in a chain and a single cell. Gametogenesis starts and two identical nonflagellated, spherical gametes appear from each gametangium. This is a cis-anisogamy, i.e. the + and − gametes do not behave in the same way: each of the two slightly mobile (active) + gametes fuse with the two immobile − (passive) gametes that are directly opposite to them, but not necessarily at the same time. Gamete fusion (plasmogamy) occurs within 1–2 min, resulting in the formation of a spherical zygote for each pair of gametes. Each zygote, which remains attached to the − parental frustule, expands to form an elongated auxospore, which is surrounded by a perizonium (Fig. 4). The initial cell develops inside the auxospore and, after 2–4 d when it reaches the maximum cell length for the species, it escapes by breaking through the tips of the perizonium (Davidovich & Bates 1998; Amato *et al.* 2005; D'Alelio *et al.* 2009). The initial cell does not have the same morphology as the vegetative cell (Kaczmarska *et al.* 2000). Curiously, initial cells of *P. multiseries*, at least, must be isolated from the parental cell mixture for them to survive (personal observation). After the first cell division, the morphology begins to appear like the normal vegetative cells.

The sexual stages are fragile and easily broken during the sampling, rendering them difficult to observe (Davidovich & Bates 1998; Mann & Bates 2001). Furthermore,

identification of these different sexual stages is difficult. Molecular probes must still be developed for identifying the sex of the cell, sexually induced cells, as well as the sexual stages (Mann & Bates 2001). In the ocean, only 9–14% of the cells, depending on the species, would be able to reproduce at the same time (Sarno *et al.* 2010). Nevertheless, two massive sexual reproduction events have recently been observed, for the first time, in natural populations (Holtermann *et al.* 2010; Sarno *et al.* 2010). These field studies, and that of D'Alelio *et al.* (2010), show a period between cycles of sexual reproduction (2–3 yr) similar to that found in culture studies (Davidovich & Bates 1998).

Questions remain as to how cells of opposite mating type find each other, and when they do, what triggers them to initiate the steps in sexual reproduction. There is some evidence that a compound is released by *P. multiseries* cells when cells of opposite mating type are placed together in 14–40 ml of medium (Haché 2000; Bates & Davidovich 2002). When cell-free filtrate collected from the medium containing the mating cells was added to other pairs of cells of opposite mating type, the number of gametes produced increased. Furthermore, some compound in that filtrate caused cells to produce gametes even in absence of any cells of the opposite mating type. Could that compound be a pheromone? The coincidence of auxosporulation occurring at the same time and location as high DA concentrations in razor clams at Kalaloch Beach (Washington, USA) (Holtermann *et al.* 2010) also raises the question about a possible relationship between sexual reproduction and production of DA.

One conclusion from the above studies is that defining a species from morphology only is inadequate; combining morphology and genetic sequencing is better (Lundholm & Moestrup 2002; Lundholm *et al.* 2002a, b, 2003, 2006), and adding mating compatibility (Amato *et al.* 2007; Lundholm *et al.* in press) provides the most complete picture (Mann 1999). For example, strains of opposite mating type and grouped together on the basis of their ITS2 sequences, which also gave the same morphological features, produced viable offspring when crossed, whereas crosses between strains of different ITS2 sequences failed to mate successfully (Amato *et al.* 2007).

Producers of DA

Domoic acid was first discovered in the red alga *Chondria armata* (Takemoto & Daigo 1958), and is also produced by two other pennate diatoms, in addition to certain *Pseudo-nitzschia* species: *Amphora coffeaeformis* from Canada (although this finding has been questioned; Bates 2000), and *N. navis-varingica* from Southeast Asia (Kotaki *et al.* 2000). In the Philippines, strains of *N. navis-varingica* from one region only produced DA, although elsewhere some isolates did produce isodomoic acids A and B (Bajarias *et al.* 2006; Kotaki *et al.* 2006).

Of the 37 species of *Pseudo-nitzschia*, 14 have been reported to produce DA, although not all strains are toxic under the testing conditions (Table 1; Bates *et al.* 1998; Thessen *et al.* 2009). *Pseudo-nitzschia* cf. *granii* is the most recent addition to the list of toxigenic species, although toxicity was demonstrated only in a shipboard continuous culture of a natural seawater sample at Ocean Station PAPA (OSP) (Trick *et al.* 2010; see below), not in a clonal isolate. In contrast, Guannel *et al.* (2011) determined that an isolate of *P. granii* from OSP was undectable for DA. They did report, however, relatively high levels of cellular DA (13.48 pg DA cell^{-1}) in an unidentified new species of *Pseudo-nitzschia* (*P.* sp. 233, strain PNWH20 233), isolated from Sequim Bay, Washington (USA). Ten species have not yet been tested for toxigenicity: *P. antarctica*, *P. fryxelliana*, *P. linea*, *P. lineola*, *P. mannii*, *P. micropora*, *P. prolongatoides*, *P. pungiformis*, *P. roundii* and *P. sinica*. Some researchers believe that all species of *Pseudo-nitzschia* will prove to be toxigenic, given the proper growth conditions and sensitive-enough detection protocols, although perhaps not at levels great enough to generate toxicity at higher trophic levels under all conditions (Parsons *et al.* 1999; Wells *et al.* 2005).

Most toxic species are coastal, with cellular DA values on the order of < 1 to ~ 100 pg cell^{-1} (although usually $<$ 100 pg cell^{-1}), depending on the species, cell size and growth conditions (laboratory studies summarized by Trainer *et al.* 2008; field studies summarized by Caron *et al.* 2010). For example, the large (73–129 µm long, 6.0–7.8 µm wide) *P. australis* contained a maximum of 78 pg DA cell^{-1} (Trainer *et al.* 2000), and the small (10–82 µm long, 1.0–1.8 µm wide) *P. galaxiae*, when toxic, contained only 0.36 fg DA cell^{-1} (Cerino *et al.* 2005). Other examples of cellular DA values, calculated from total abundances of *Pseudo-nitzschia* spp. and particulate DA concentrations in the field, are: 2.7 pg cell^{-1} (Adams *et al.* 2000), 88 pg cell^{-1} (Anderson *et al.* 2009) and 117 pg cell^{-1} (Schnetzer *et al.* 2007); 43 pg cell^{-1} was reported for *P. australis* (Howard *et al.* 2007).

There are few oceanic species: *P. turgidula*, *P. heimii*, *P. inflatula*, *P. granii* and *P. prolongatoides*, although the first three are also found along some coasts (Rhodes *et al.* 1996; Priisholm *et al.* 2002; Hernández-Becerril & Díaz-Almeyda 2006; Congestri *et al.* 2008). Previously, none of the species isolated from mid-ocean waters was reported to produce DA at detectable levels (Marchetti *et al.* 2008); *P. prolongatoides* has not yet been tested (Table 1). However, a recent study carried out in the eastern subarctic Pacific (at OSP) has shown exceedingly low levels of DA in seawater (0.1 pg DA ml^{-1}) and in *P.* cf. *granii* cells (4 ag DA cell^{-1}) from a shipboard growth experiment that used a natural seawater sample (Trick *et al.* 2010). By comparison, coastal waters contained much greater concentrations, ranging from 0.15–9.39 ng DA ml^{-1} in San Francisco Bay (Howard *et al.* 2007) to 136 ng DA ml^{-1} in Washington State (Trainer *et al.* 2007). The above oceanic studies are among many that have been carried out to examine the effects of adding iron to stimulate the growth of iron-limited phytoplankton in the high-nitrate, low-chlorophyll (HNLC) regions of mid-oceans. It has been proposed that the phytoplankton growth stimulated by this artificial fertilization could result in a drawdown of atmospheric CO_2 concentrations and hence a slowing of global warming, although the effectiveness of this approach has recently been questioned (Hamme *et al.* 2010). Moreover, it has raised concerns because numerous tests have resulted in favouring the growth of *Pseudo-nitzschia* spp. (summarized

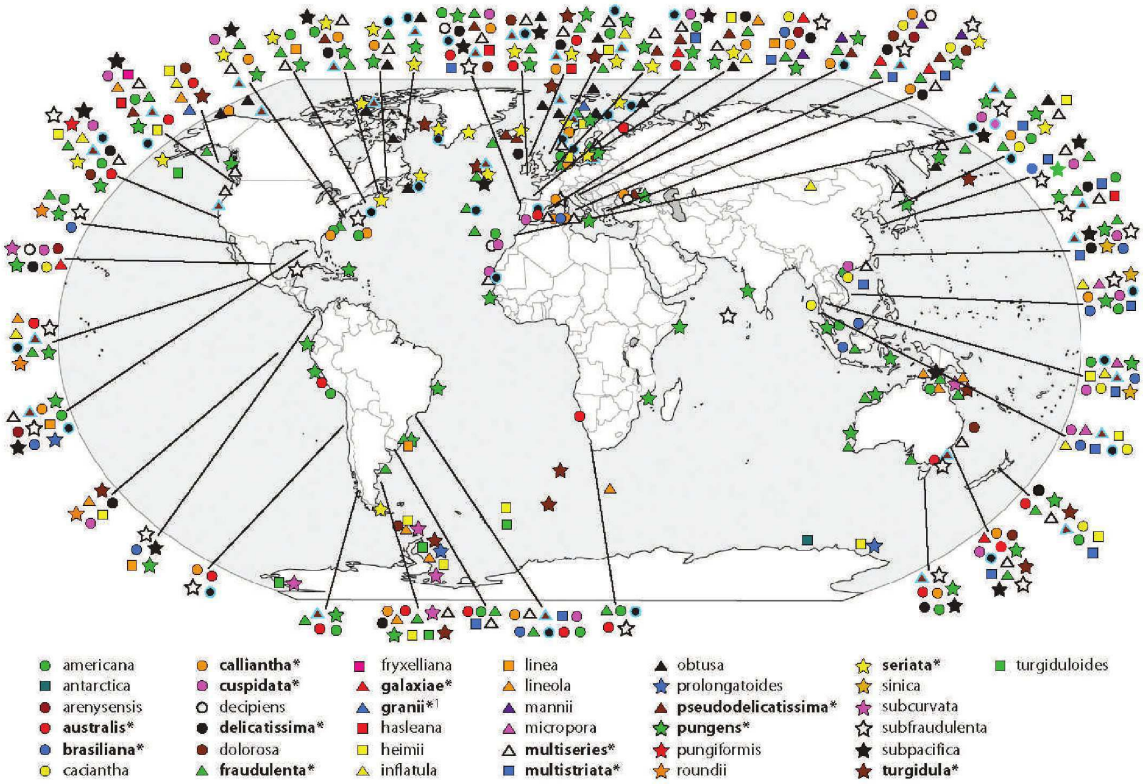


Fig. 11. World distribution of *Pseudo-nitzschia* spp. (see also Table 1). Toxigenic species are in bold and shown with an *; note that only certain strains of these species are toxigenic at some locations.[1] Toxicity determined from a shipboard continuous culture of a natural seawater sample containing *P.* cf. *granii* as the only species of *Pseudo-nitzschia* (Trick *et al.* 2010). Symbols outlined in blue indicate that the report was made before major taxonomic revisions were made for *P. delicatissima* and *P. pseudodelicatissima*, although updates were made when available. Modified and updated from Thessen (2007). A color version of this map is available online.

by Trick *et al.* 2010). Furthermore, Trick *et al.* (2010) showed that iron addition stimulated not only *Pseudo-nitzschia* spp. growth, but also the production of DA by *P.* cf. *granii* (see above) and *P. turgidula* (see more on iron stimulation, below). Although the amounts of toxin produced are low (4 ag DA cell^{-1} and 0.3 fg DA cell^{-1} for *P.* cf. *granii* and *P. turgidula*, respectively), there are still unknown consequences of this to the ocean ecosystem.

Blooms

Diatoms, including *Pseudo-nitzschia* spp., often bloom in upwelling zones, where currents allow them to remain in the upper, sunlit water column and to take advantage of the rich nutrients coming from the ocean depths. For example, *Pseudo-nitzschia* blooms are common along the west coast of the United States (Horner *et al.* 2000; Trainer *et al.* 2000; Kudela *et al.* 2005; Anderson *et al.* 2006), where river runoff can also supply nutrients (Schnetzer *et al.* 2007), although runoff is not always implicated (Kudela *et al.* 2004a). Worldwide, *Pseudo-nitzschia* blooms are also common along the west coast of continents because of upwelling as well as to circulation patterns caused by seafloor and coastal topographies (Trainer *et al.* 2008). This can cause them to be retained for long periods under conditions that are favourable for elevated DA production, e.g. in the Juan de Fuca eddy region off the coasts of Washington (USA) and British Columbia (Canada) (Trainer *et al.* 2009a) and in the eddy of the Santa Barbara Channel, California (USA) (Anderson *et al.* 2006). Otherwise, *Pseudo-nitzschia* spp. can be found in other coastal or mid-oceanic waters (Trainer *et al.* 2008), as well as in cold (polar) (Orlova & Shevchenko 2002; Almandoz *et al.* 2008), temperate (Amato *et al.* 2005) or tropical/subtropical waters (Hernández-Becerril 1998; Hasle 2002) (Table 1). *Pseudo-nitzschia* spp. are found on all continents, including Antarctica, although no toxigenic species have yet been found there (Fig. 11). Since last reported in 2007, the number of species and locations documented has increased; compare the distribution map in Thessen (2007) with that shown in Fig. 11. The increases are for all continents except Antarctica, but it appears that they are usually associated with locations where research institutions devote particular attention to this genus.

The increased interest directed at *Pseudo-nitzschia* spp., and the latest naming of new species, has resulted in their recent discovery in new locations (Table 2). Some species are described as being cosmopolitan: *P. australis*, *P. delicatissima*, *P. fraudulenta*, *P. multiseries*, *P. pseudodelica-*

Table 2. New records for *Pseudo-nitzschia* spp. at different locations of the world, since 2002.

Species	Location	Reference
P. americana	Russia (NW Sea of Japan, Sea of Okhotsk)	Orlova & Shevchenko 2002
	Bay of Fundy	Kaczmarska *et al.* 2005b
	Atlantic coast of France	Nézan *et al.* 2007
	Portugal	Churro *et al.* 2009
	Denmark and Scandinavia	Lundholm *et al.* 2010
P. brasiliana	Mediterranean coast of Spain	Quijano-Scheggia *et al.* 2005
	Tokyo Bay, Japan	Yap-Dejeto *et al.* 2010
	Eastern Russian seas	Stonik *et al.* 2011
	Bizerte Lagoon, Tunisia	Sahraoui *et al.* in press
P. caciantha	Tokyo Bay, Japan	Yap-Dejeto *et al.* 2010
P. calliantha	Turkish coast of the Black Sea	Bargu *et al.* 2002a
	southern Adriatic coastal waters	Caroppo *et al.* 2005
	Chesapeake Bay	Thessen & Stoecker 2008
	Black Sea[1]	Besiktepe *et al.* 2008
	Bizerte Lagoon, Tunisia	Sahraoui *et al.* 2009
	northern Chile	Álvarez *et al.* 2009
	Portugal	Churro *et al.* 2009
	Gulf of Mexico	Del Rio *et al.* 2010
	Tokyo Bay, Japan	Yap-Dejeto *et al.* 2010
P. cuspidata[2]	coastal Washington State	Trainer *et al.* 2009b
	Portugal	Churro *et al.* 2009
P. delicatissima	southern Adriatic coastal waters	Caroppo *et al.* 2005
	Greece	Moschandreou *et al.* 2010
P. dolorosa[3]	Drake Passage	Ferrario *et al.* 2004
	Greece	Moschandreou *et al.* 2010
P. fraudulenta	Bay of Fundy	Kaczmarska *et al.* 2005b
	Russian waters of the Japan Sea	Stonik *et al.* 2008
	Greece	Moschandreou *et al.* 2010
P. galaxiae	Greece, Spain (Mediterranean Sea)	Moschandreou *et al.* 2010; Quijano-Scheggia *et al.* 2010
	France (Atlantic)	Orive *et al.* 2010
	Tokyo Bay, Japan	Yap-Dejeto *et al.* 2010
P. heimii	Argentina	Almandoz *et al.* 2007
	Denmark and Scandinavia	Lundholm *et al.* 2010
P. inflatula	Mexico (Pacific)	Hernández-Becerril & Díaz-Almeyda 2006
	Tyrrhenian Sea (Italy)	Congestri *et al.* 2008
P. linea	Mediterranean Sea (Spain)	Quijano-Scheggia *et al.* 2010
	Paraná coast, Brazil	Fernandes & Brandini 2011
P. lineola	Argentina	Almandoz *et al.* 2007
P. mannii	Greece	Moschandreou *et al.* 2010
P. multiseries	Bay of Fundy	Kaczmarska *et al.* 2005b
P. multistriata	Mediterranean coast of Spain	Quijano-Scheggia *et al.* 2005
	Uruguay	Méndez & Ferrario 2009
	Portugal	Churro *et al.* 2009
	Greece	Moschandreou *et al.* 2010
	Sea of Okhotsk (Russia)	Stonik *et al.* 2011
P. pseudodelicatissima	Denmark and Scandinavia	Lundholm *et al.* 2010
	Greece	Moschandreou *et al.* 2010
P. pungens	Bay of Fundy	Kaczmarska *et al.* 2005b
P. seriata	west coast of Greenland	Hansen *et al.* 2011
	Eastern Russian seas	Stonik *et al.* 2011
	Greece	Moschandreou *et al.* 2010
P. subfraudulenta	northern Chile	Álvarez *et al.* 2009
	Greece	Moschandreou *et al.* 2010
P. subpacifica	Bay of Fundy	Kaczmarska *et al.* 2005b
	Portugal	Churro *et al.* 2009
P. turgidula	Bay of Fundy	Leandro *et al.* 2010a
P. turgiduloides	Argentina	Almandoz *et al.* 2007

[1] Lundholm *et al.* (2003) previously reported that the *P. pseudodelicatissima* used by Davidovich & Bates (1998) and isolated from the Black Sea was actually *P. calliantha*.
[2] Previously reported as *P.* cf. *pseudodelicatissima* in these waters (e.g. Marchetti *et al.* 2004).
[3] Indicated as 'one strain similar to *P. dolorosa*' by Moschandreou *et al.* (2010).

tissima and *P. pungens* (Hasle 2002; Lundholm & Moestrup 2002), but Table 1 and Fig. 11 suggest that most of the others could also be in that category, except for: *P. antarctica*, *P. arenysensis*, *P. fryxelliana*, *P. granii*, *P. mannii*, *P. micropora*, *P. prolongatoides*, *P. pungiformis*, *P. roundii*, *P. subcurvata* and *P. turgiduloides*, which have a more restricted distribution, including polar regions. As well, some of the cosmopolitan species may actually be more diverse, if cryptic species are considered. It is not yet clear if the distribution of the noncosmopolitan species is in fact caused by an inability to grow in a broad spectrum of conditions, or if they are truly stenohaline or stenothermal

(see below). Interestingly, all of noncosmopolitan species have so far been shown to be nontoxic (Table 1), which supports the hypothesis that the cosmopolitan species are toxigenic (Hasle 2002). Curiously, although *P. australis* is listed as a cosmopolite (Hasle 2002), it is absent from the northwestern Atlantic (east coasts of the United States and Canada). A worldwide distribution may be explained by ballast water transport, relocation of aquacultured bivalve molluscs and a greater adaptive ability of some species (Zhang & Dickman 1999; Burkholder *et al.* 2007; Hégaret *et al.* 2008). Two recent reports of an unexpected presence of a *Pseudo-nitzschia* species require further investigation: *P.* cf. *subcurvata* (identified by light microscopy only, so not shown in Fig. 11), normally in polar regions (Fryxell *et al.* 1991; Bates *et al.* 1993a; Almandoz *et al.* 2007, 2008), was reported along the Mexican coast of the Gulf of Mexico (Aké-Castillo & Okolodkov 2009), and *P. seriata*, normally in the North Atlantic Ocean (Hasle 2002; Hasle & Lundholm 2005) and recently in eastern Russian seas (Stonik *et al.* 2011), was reported in the Beagle Channel of Argentina (Almandoz *et al.* 2009), although it exhibited only two rows of poroids. It should be pointed out that a strain of *Pseudo-nitzschia* isolated from Puget Sound, Washington (USA) was reported to be *P. seriata* on the basis of its morphometrics and ITS1 sequences (Hubbard *et al.* 2008).

Pseudo-nitzschia spp. are a frequent part of the phytoplankton community. Several species of *Pseudo-nitzschia* often grow at the same time and location, e.g. in the Bay of Fundy (Canada), where seven species are found, among them five potentially toxic (Kaczmarska *et al.* 2005b) (Table 1). Nine species are found in Monterey Bay, California, two of which are toxigenic (*P. australis*, *P. multiseries*; Kudela *et al.* 2004b) and one (*P. turgidula*) potentially so. Seven species are found in coastal Washington waters; all except possibly *P.* cf. *heimii* are toxigenic (Stehr *et al.* 2002). Likewise, seven *Pseudo-nitzschia* spp. are in Chesapeake Bay (Marshall *et al.* 2005; Thessen & Stoecker 2008), only one of which (*P. subpacifica*) has never been proven to be toxigenic. Ten species of *Pseudo-nitzschia* grow in the Gulf of Naples, although some during different seasons (Orsini *et al.* 2002; Lundholm *et al.* 2003; Cerino *et al.* 2005; Amato *et al.* 2007; McDonald *et al.* 2007; D'Alelio *et al.* 2009). Only *P. multistriata* (Orsini *et al.* 2002) and *P. galaxiae* (Cerino *et al.* 2005) are toxigenic, but with low cellular DA content (0.8 ag DA cell^{-1} for *P. galaxiae*) because of their small cell size, thus possibly explaining the absence of any ASP events in Italian waters. Six species were documented over an entire year in Normandy (France), with *P. australis* as the most important toxigenic diatom (Klein *et al.* 2010).

Where there are sufficient long-term data on *Pseudo-nitzschia* spp. and accompanying environmental information, it has been possible to develop predictive models of toxigenic *Pseudo-nitzschia* blooms, e.g. the northwest coast of the Iberian Peninsula, Spain (Corchado *et al.* 2004); Monterey Bay [Lane *et al.* 2009; although the presence of *P. australis* in thin layers (McManus *et al.* 2008) is one challenge for applying the model]; Santa Barbara Channel, California (Anderson *et al.* 2009); Chesapeake Bay (Anderson *et al.* 2010); and Lisbon Bay, Portugal (Palma *et al.* 2010). Improvements are still required to predict more accurately the timing of a bloom's appearance and its intensity. Commonalities of the above locations are that they are either in upwelling zones or in coastal bays, with high levels of macronutrients and perhaps trace metals (Trainer *et al.* 2008, and see below).

Shifts in the relative abundance of *Pseudo-nitzschia* spp. have been documented on seasonal, decadal and centennial timescales. Different species of *Pseudo-nitzschia* have unique seasonal distributions, often linked to temperature and salinity (Klein *et al.* 2010), although some may be present all year at the same location (Hasle *et al.* 1996). Blooms mainly occur between January and May in European waters, rarely during summer (Hasle *et al.* 1996); in the fall in eastern North America (Bates *et al.* 1998); early summer (Trainer *et al.* 2002) or early fall in Washington State (Trainer *et al.* 2010); or late spring in Southern California (Anderson *et al.* 2006, 2009) and the Pacific Mexican coast (García-Mendoza *et al.* 2009). There is sometimes a seasonal succession of *Pseudo-nitzschia* spp., e.g. *P. pungens* has tended to bloom in late summer waters, before *P. multiseries* in eastern and western Canadian waters, and in the Gulf of Mexico (Bates *et al.* 1998). In Scottish waters (Fehling *et al.* 2006), blooms of *P. pungens*, *P. pseudodelicatissima* and *P. australis* are more likely to occur during the warm season, whereas blooms of *P. multiseries* tend to occur during the cold season (late fall and spring). In the Bay of Naples, blooms of *P. galaxiae* occur between February and November, with higher concentrations in May and August (Cerino *et al.* 2005). Numbers of *Pseudo-nitzschia* species in Peter the Great Bay (eastern Russia) peak at the summer and fall (Stonik *et al.* 2011). In the Argentine Sea, *P. pungens* and *P. australis* reach high densities at warm temperatures (15.8°C), high salinities [33.8 practical salinity units (psu)] and low nutrient concentrations (Almandoz *et al.* 2007). *Pseudo-nitzschia heimii*, *P. lineola*, *P. turgidula* and *P. turgiduloides* are more restricted in distribution and reach lower cell densities. They are associated with low salinity (32.45 psu) and cold (8.8°C) waters that are rich in nutrients (Almandoz *et al.* 2007). In contrast to the above, *P. pungens* was present all year in Normandy (France), indicating that it exhibited lower environmental constraints than the other local *Pseudo-nitzschia* species (Klein *et al.* 2010).

On a decadal timescale, there was a shift in the Skagerrak from *P. multiseries* during 1967–1968 to *P. pungens* in the 1970s and 1980s. This was speculated to be caused by warmer waters that favoured the latter (Hasle 1995), in agreement with the above seasonal patterns. In the southern Gulf of St. Lawrence (Bates *et al.* 1998), and perhaps elsewhere in the world, *P. multiseries* abundance has also drastically decreased since *c.* 1990, to the point that it has become more difficult, recently, to obtain fresh isolates of this species in the field (personal observation). Further work is required to determine if this decline correlates with the worldwide increasing trend in sea surface temperatures over the past century, which is also linked to the global decline of phytoplankton biomass (Boyce *et al.* 2010). A study of species composition along the California coast during 2000–2006 discovered a decrease in *P. multiseries* and *P. australis*, as well as a significant decline in shellfish contaminated with DA, after 2004, when the species composition shifted toward toxic *Alexandrium* and *Dino-*

physis (Jester *et al.* 2009). However, after 2007, toxigenic *Pseudo-nitzschia* spp. again became dominant, with the exception of *P. multiseries*, along with a recurrence of DA contamination (Jester *et al.* 2009).

On a centennial timescale, one dominant species has been shown to replace another. The sediment record showed that *P. multiseries* in Mariager Fjord (Denmark) made up > 90% of the *Pseudo-nitzschia* cells from 1905 to 1947, but then it decreased dramatically to ~ 5% in 2002 (Lundholm *et al.* 2010). It was replaced by *P. pungens*, which increased in relative abundance from < 1% in the period 1905–1947 to 63% in *c.* 1964. Consistent with the above, because *P. pungens* tends to be favoured by warmer temperatures in the field (but supported by only some laboratory experiments; see below), Lundholm *et al.* (2010) hypothesized that the increase in seawater temperature observed over the last century could have affected the competitive balance between the two species.

Pseudo-nitzschia spp. are sometimes a large proportion of the total diatom biomass. The contribution of the *seriata* group species reached 77% in western Scottish waters; that for the *delicatissima* group was 14% (Fehling *et al.* 2006). In those waters, there was a negative correlation between *Pseudo-nitzschia* abundance and concentrations of nitrogen, phosphorus and silicon, with a depletion of each before the bloom reached its maximum. Each species, whatever its group, can be linked to different physicochemical factors (Kaczmarska *et al.* 2007; Schnetzer *et al.* 2007; Almandoz *et al.* 2008). For example, in Scottish waters, the *seriata* group species are linked to temperature, whereas the *delicatissima* group species are linked to salinity and the presence of ammonium (Fehling *et al.* 2006). Photoperiod can influence the timing of *Pseudo-nitzschia* blooms and showed a strong positive correlation with their presence (Fehling *et al.* 2006). It also affects sexual reproduction (see above; Hiltz *et al.* 2000).

Pseudo-nitzschia spp. may be located throughout the water column or concentrated in layers. Healthy, motile chains of *P. fraudulenta* were found in thin layers at depths of ~ 5 m and ~ 24 m, in an estuarine fjord (Washington State); these were likely advected from the surface (Rines *et al.* 2002). High concentrations of *P. australis* were found in a narrow band at 10–20-m depth, in Monterey Bay, California (Ryan *et al.* 2005). Thin layers of *P.* cf. *pseudodelicatissima* and *P. australis*, likely senescent and therefore containing DA, were also found at 10–20 m, in a Galician Ría, Spain (Velo-Suárez *et al.* 2008). Such layers may be missed by conventional monitoring methods. All were just above or within the pycnocline and may be displaced vertically during downwelling events, allowing them to contaminate benthic organisms (Velo-Suárez *et al.* 2008). Single cells, apparently dead, were found in sediment traps at 10–16 m in the Gulf of Mexico (Dortch *et al.* 1997). Cellular DA was found in sediment traps below the euphotic zone in coastal California waters (Trainer *et al.* 2000). Particulate DA, chlorophyll-containing cells and even intact chains were found sinking at depths of up to 800 m off the coast of Southern California (Schnetzer *et al.* 2007; Sekula-Wood *et al.* 2009, 2011). Such cells may be a source of DA that contaminates benthic communities (Kvitek *et al.* 2008; see below). These cells may also be a seed population if brought to the surface during the next upwelling event (Trainer *et al.* 2000; Mengelt & Prézelin 2002).

PHYSICAL PARAMETERS AFFECTING GROWTH AND TOXICITY

Growth phase

DA production is associated with different phases of the *Pseudo-nitzschia* growth cycle, perhaps linked to changes in physicochemical parameters of the surrounding medium caused by the cells' growth. Early studies with *P. multiseries* and a Danish strain of *P. seriata* showed that DA production started in late-exponential phase and continued more rapidly during the stationary phase (summarized by Bates 1998). This was confirmed by Fehling *et al.* (2004) in another strain of *P. seriata*. Likewise, *P. cuspidata* (Trainer *et al.* 2009b), *P. fraudulenta* and *P. calliantha* (Thessen *et al.* 2009) produced most of its DA during the stationary phase, although some was reported during the exponential phase. Interestingly, *N. navis-varingica* showed this same pattern (Kotaki *et al.* 2000). In contrast to the above, DA was produced by *P.* sp. cf. *pseudodelicatissima* during the early exponential phase (Pan *et al.* 2001). Few studies have since been carried out to examine the timing of DA production in batch culture, and existing results are sometimes contradictory. In the case of *P. pseudodelicatissima*, this may perhaps be explained by the recent finding that this species is actually composed of at least seven species (see above), some of which may behave differently. For example, the *P.* sp. cf. *pseudodelicatissima* mentioned above could be identified as either *P. pseudodelicatissima* or *P. cuspidata* (Lundholm *et al.* 2003). Likewise, the true identity of '*P. pseudodelicatissima*' from Washington State coastal waters is not known with certainty; it produced DA steadily through the exponential phase, and then production increased dramatically during the late-stationary phase (Adams *et al.* 2000). Garrison *et al.* (1992) showed that *P. australis* produced DA during most of the exponential phase but not during stationary phase. In contrast, Cusack *et al.* (2002) documented that *P. australis* began DA production in late-exponential phase and it continued into stationary phase. Interestingly, under conditions of silicate or phosphate limitation, the cells release increasing proportions of produced DA into the growth medium during stationary phase (reviewed by Bates 1998). Additional species require study to verify these patterns. In the end, it must still be resolved if changes in DA production over the growth cycle are due to some intrinsic alteration in the cells' physiology during growth, or to a specific nutrient limitation per se (see below).

Over a longer timescale, the viability and toxicity of *P. multiseries* (Bates 1998), *P. australis* (Rhodes *et al.* 2004) and perhaps other species as well decline over a period of several years in culture. For several strains of *P. multiseries*, the decrease in cellular toxicity was exponential and was associated with a reduction in cell length over a 2.7-yr period, resulting in a positive linear relationship between cellular DA and cell volume (Mafra *et al.* 2009a). As discussed above, a cell decreases to a species-specific length before it is capable of rejuvenating its large cell size via sexual

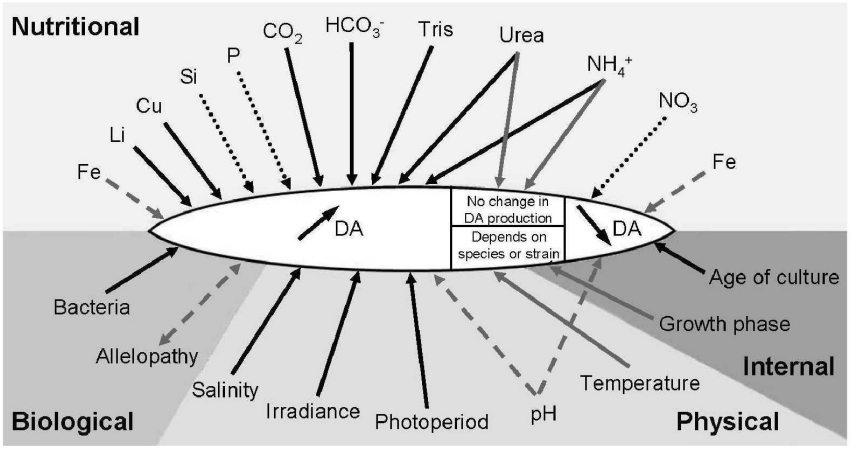


Fig. 12. Synthesis of all the factors (nutritional, internal, physical or biological) studied and their impacts on domoic acid (DA) production (increase or decrease). Black arrow = effect of increasing the parameter; black dotted arrow = effect of decreasing the parameter; grey dashed arrow = conflicting results found. Grey arrow = result depends on species or strain, or no change observed in DA production.

reproduction. Large cells, just after sexual reproduction, produced more DA than their smaller-celled parents, once they had first divided several times (Bates *et al.* 1999). Some, but not all, offspring of *P. cuspidata* were also more toxic than their parents (Lundholm *et al.* in press). There may thus be a relationship between cell toxicity and readiness to undergo sexual reproduction, but this has not been proven. Interestingly, the cellular DA content of *P.* sp. cf. *pseudodelicatissima* increased by an order of magnitude after 'cell enlargement' (apparently not related to sexual reproduction) (Pan *et al.* 2001). The decline in ability to produce DA may also be related to a gradual decline in cellular chlorophyll *a*, which provides photosynthetic energy required for DA biosynthesis (Pan *et al.* 1998), although this requires more study. Another hypothesis is that it could be related to changes in the bacterial assemblage growing with *Pseudo-nitzschia* (Bates 1998). Stewart (2008) proposed that the presence of DA-degrading bacteria could explain the decline over months in culture. Differences were found in the bacterial community composition over time in culture, although for nontoxic *P. pungens*, which supports this possibility (Sapp *et al.* 2007). On the other hand, Guannel *et al.* (2011) found no shifts in bacterial assemblages in a *Pseudo-nitzschia* culture over its initial 9 mo (see below).

Results of any study that used only one strain of a given *Pseudo-nitzschia* species to evaluate a factor affecting DA production must be interpreted with caution, because of the high variability among strains of the same species (Bates *et al.* 1999; Kudela *et al.* 2004b; Thessen *et al.* 2009; Amato *et al.* 2010; Lundholm *et al.* in press). This was the case in most of the studies below, unless otherwise indicated. A summary of factors affecting DA production is given in Table 3 and Fig. 12.

Temperature

Laboratory studies, up to about 1998, on the effects of temperature on DA production, growth and photosynthesis of *P. multiseries* are reviewed in Bates (1998); *P. pseudodelicatissima* (Lundholm *et al.* 1997) was not included in that review. Additional species have since been studied: *P. cuspidata*, *P. granii* and *P. pungens* (Table 4), but this is still a small fraction of the 37 known species. As well, only one strain of each species has been studied.

Acclimatization time (~ 7 d: Miller & Kamykowski 1986; Lundholm *et al.* 1997; 10 generations: El-Sabaawi & Harrison 2006), interactions between temperature and salinity (Miller & Kamykowski 1986; Lundholm *et al.* 1997; Doan-Nhu *et al.* 2008), as well as irradiance (El-Sabaawi & Harrison 2006) must be considered when temperature characteristics are measured. This is because growth at different temperatures is salinity dependent. For example, *P. cuspidata* tolerated a wider range of temperatures when grown at its optimum salinity (30 psu) (Doan-Nhu *et al.* 2008). Likewise, *P. pseudodelicatissima* achieved the highest growth rate at 25°C when measured at its optimum salinity for growth (25‰), although higher temperatures were not tested (Lundholm *et al.* 1997). Furthermore, this species reached a lower temperature limit for growth (5°C) at this optimum salinity than at any other salinity. Similar findings were reported for *P. americana*, whose optimum growth temperature (25 ± 2.5°C) was salinity dependent (Miller & Kamykowski 1986). This species was unable to grow at a low salinity (8‰), even at its optimum growth temperature.

The growth responses from individual laboratory experiments tend to support the temperature ranges found in the waters from which the *Pseudo-nitzschia* spp. were isolated. For example, the ability of *P. multiseries* to grow at a lower temperature than *P. pungens* in culture (Table 4) can explain its higher cell concentration at lower temperatures in the fall and spring in Chinhae Bay (South Korea) (Cho *et al.* 2001). Likewise, *P. multiseries* was more likely to be abundant at lower temperatures than *P. pungens* in Prince Edward Island (Canada) and elsewhere (Bates *et al.* 1998). The growth response of *P. cuspidata* at high temperatures (Table 4) and salinities (Table 5) may account for its presence under these conditions in Nha Trang Bay

Table 3. Summary of factors affecting the production of domoic acid (DA) by *Pseudo-nitzschia* spp. in culture; modified and updated from Mafra (2009).

Factor	Species	Effect on DA production (range in growth parameter)	Reference
Physicochemical			
Temperature	*P. seriata*	DA higher at 4°C than at 15°C; 1.0–33.6 pg cell^{-1}, 0.31–1.6 pg cell^{-1}, respectively	Lundholm *et al.* 2004
	P. multiseries	increased DA (5–25°C); 100 times more at 25°C	Lewis *et al.* 1993
Salinity	*P. multiseries*	increased DA (10–40 psu); three to four times more at highest salinity	Doucette *et al.* 2008
Irradiance	*P. multiseries*	increased DA (35–130 µmol photons m^{-2} s^{-1}); seven times more DA at highest irradiance	Bates 1998
	P. australis	increased DA (11.7 and 115 µmol photons m^{-2} s^{-1}); 24–130 times more at the higher irradiance	Cusack *et al.* 2002
Photoperiod	*P. seriata*	increased DA (18:6 L:D cycle compared with 9:15 L:D); 1.5 times more with longest photoperiod	Fehling *et al.* 2005
pH	*P. multiseries*	increased DA at higher pH (7.9–8.9); 74 times more at highest pH	Lundholm *et al.* 2004; Trimborn *et al.* 2008
	P. multiseries	increased DA at lower pH (8.38–7.94); 1.4–5 times more at lower pH	Sun *et al.* 2011
Nutritional			
Silica	*P. multiseries*	produced DA under Si limitation	Bates *et al.* 1991; Pan *et al.* 1996b, c; Kudela *et al.* 2004b; Hagström *et al.* 2010
	P. sp. cf. *pseudodelicatissima*	produced DA under Si limitation	Pan *et al.* 2001
	P. australis	produced DA under Si limitation	Cusack *et al.* 2002
	P. cuspidata	produced DA under Si limitation	Trainer *et al.* 2009a
	P. seriata	produced DA under Si limitation	Fehling *et al.* 2004; Kudela *et al.* 2004b
Phosphorus	*P. multiseries*	produced DA under P limitation	Bates *et al.* 1991; Pan *et al.* 1996a
	P. seriata	produced DA under P limitation	Fehling *et al.* 2004
Nitrate	*P. multiseries*	no detectable DA under N deficiency	Bates *et al.* 1991
	P. multiseries	low DA in N-limited chemostat cultures; 1000 times less than in Si-limited chemostats	Kudela *et al.* 2004b
	P. multiseries	low DA in N-deficient batch culture; 2.5 times less than urea, 15 times less than nitrate, but reported only on 1 d	Calu *et al.* 2009
	P. australis	increased DA (50 µM added)	Howard *et al.* 2007
Ammonium	*P. multiseries*	increased DA (110–440 µM added); two to four times more than at 55 µM and the same concentration of nitrate	Bates *et al.* 1993b
	P. multiseries	increased DA in some strains (88 µM added)	Thessen *et al.* 2009
	P. calliantha	increased DA in some strains (88 µM added)	Thessen *et al.* 2009
	P. fraudulenta	increased DA in some strains (88 µM added)	Thessen *et al.* 2009
	P. australis	increased DA (10 µM added)	Howard *et al.* 2007
Urea	*P. australis*	increased DA (20 µM added); two times more DA than nitrate, three times more than ammonium and control treatments	Howard *et al.* 2007
	P. multiseries	increased DA (220 µM added); ~ two times more than nitrate on day 8 in batch culture, then the same	Calu *et al.* 2009
	P. pungens	increased DA (220 µM added); ~ two times more than nitrate in chemostat culture	Calu *et al.* 2009
	P. multiseries	increased in some strains (88 µM added)	Thessen *et al.* 2009
	P. calliantha	increased in some strains (88 µM added)	Thessen *et al.* 2009
	P. fraudulenta	increased in some strains (88 µM added)	Thessen *et al.* 2009
Tris buffer	*P. multiseries*	increased DA (2.1–8.2 mM added); two to three times more than the control with no Tris added	Douglas *et al.* 1993
Bicarbonate	*P. multiseries*	increased DA (1–2 mM added); 4.8 times and 11.5 times more when supplemented with 1 and 2 mM bicarbonate, respectively	Bates & Léger 2006
Carbon dioxide	*P. multiseries*	increased DA (220–730 ppm added); 1.4 times and 5.0 times more when supplemented with 400 ppm and 730 ppm CO_2, respectively	Sun *et al.* 2011
Lithium	*P. multiseries*	increased DA (386 µM added); 1.7 times compared with control	Subba Rao *et al.* 1998

Table 3. Continued

Factor	Species	Effect on DA production (range in growth parameter)	Reference
Iron	*P. multiseries*; *P. australis*	increased DA under Fe limitation, during late-exponential phase	Rue & Bruland 2001; Maldonado *et al.* 2002; Wells *et al.* 2005
	P. multiseries	decreased DA under Fe deficiency, during stationary phase	Bates *et al.* 2001
Copper	*P. multiseries*; *P. australis*	increased DA at high Cu concentration, during late-exponential phase	Rue & Bruland 2001; Maldonado *et al.* 2002; Wells *et al.* 2005
Biological			
Allelopathy	–	not studied specifically to test for DA production	Subba Rao *et al.* 1995; Lundholm *et al.* 2005b
Bacteria	*P. multiseries*	DA increased in the presence of bacteria	Douglas *et al.* 1993; Bates *et al.* 1995a, b, 2004; Kobayashi *et al.* 2009

(Vietnam) (Doan-Nhu *et al.* 2008). Nevertheless, *P. cuspidata* was also found in colder (12 to 15°C) upwelling waters of coastal Washington State (Trainer *et al.* 2009b). Thus, it appears that the origin of the isolate may be important, and generalizations about growth temperature should be made with caution.

As well, because *Pseudo-nitzschia* spp. have such a broad tolerance for temperature, found in nature from −1.5 to at least 30°C (summarized by Bates *et al.* 1998), temperature can be used only in a general way to elucidate species distributions. The cosmopolite nature of several *Pseudo-nitzschia* spp. (Table 1; Hasle 2002) may be explained in part by their wide temperature tolerance. In contrast, polar, tropical and oceanic species likely have different temperature optima and a narrower tolerance; e.g. *P. granii*, the only polar *Pseudo-nitzschia* species studied, has a lower optimum temperature (~ 14°C) than the other species (Table 4) and is also found in colder waters (Table 1). Its ability to grow at lower temperatures is related to an increased cellular chlorophyll *a* level (El-Sabaawi & Harrison 2006).

As more growth temperature data are obtained from laboratory studies, it is becoming clearer that there is a ~ 10°C overlap in the temperature tolerance of some *Pseudo-nitzschia* spp. (Table 4). It has thus become more difficult to correlate seawater temperature with species succession (see above). Nevertheless, temperature has been correlated with the presence of certain species in the field, e.g. a negative correlation for *P. calliantha* in the southern Adriatic Sea (Italy) (Caroppo *et al.* 2005) and Chesapeake Bay (USA) (Thessen & Stoecker 2008), and a positive correlation for *delicatissima* group species in Bizerte Lagoon (Tunisia) (Sahraoui *et al.* 2009) and for *P. americana* and *P. australis* in Normandy (France) (Klein *et al.* 2010). These findings often simply reflect that the species is found under summer or winter conditions.

Bates (1998) summarized earlier studies for five species of *Pseudo-nitzschia*, showing that DA production generally increases with increasing temperature, as expected. No further studies have been carried out to advance this area of research.

Salinity

Up to 1998, three species had been studied with respect to salinity tolerance for growth (Bates 1998): *P. multiseries*, *P. pungens* and *P. pseudodelicatissima*. Since then, additional data have become available for these species, and three more *Pseudo-nitzschia* spp. have been studied (Table 5): *P. delicatissima*, *P. cuspidata* and *P. multistriata*; *P. americana* (Miller & Kamykowski 1986) and *P. pseudodelicatissima* (Lundholm *et al.* 1997) were studied earlier but were not included in Bates (1998). All are estuarine/coastal species. Most studies were carried out at one temperature only, although as the above has shown, temperature and salinity interact to affect the growth response. For example, *P. cuspidata* can tolerate a wider range of salinities when grown at higher temperatures (Doan-Nhu *et al.* 2008), illustrating the importance of including several temperatures when studying the growth response to salinity. As well, the acclimation time to the different salinities before the growth measurements is important (Thessen *et al.* 2005). For example, a gradual acclimation resulted in a wider salinity growth range for *P. pungens* and *P. multistriata* than when the cells were shocked with a sudden change in salinity (Villac *et al.* 2004), although abrupt salinity changes may indeed occur in nature, e.g. at the mouth of a river. An ability to persist at low salinities, even without growing, is also an important aspect that is often not considered in these laboratory studies (Thessen *et al.* 2005).

The new studies add increasing evidence that these species are euryhaline and halotolerant. In the natural environment they are able to grow at salinities ranging from 1 to 39.8 psu (Thessen *et al.* 2005), e.g. with a maximum abundance between 10 and 20 psu in the Chesapeake Bay (Anderson *et al.* 2010) and 22 and 28 psu in Louisiana–Texas coastal waters (Thessen *et al.* 2005). The salinity range was slightly higher (26–32 psu) in Alabama coastal waters, where the maximum in the mean population density of *Pseudo-nitzschia* spp. occurred at 30.1 ± 3.2 psu (Liefer *et al.* 2009). When all the laboratory studies are examined there appears to be little difference between most species with respect to salinity, because of the broad range in salinity tolerance for each species (Table 5). Exceptions are *P. delicatissima*, which can grow at lower salinities and *P. cuspidata*, which grows less well at low salinities.

The salinity ranges determined in these studies are often broader than those actually found where the species are growing in nature, especially at the high end. Thus, one cannot necessarily use these laboratory studies to define when and where a particular species will bloom. Neverthe-

Table 4. Summary of temperature data (°C), showing the minimum (Min), maximum (Max) and the optimum temperature at which the *Pseudo-nitzschia* spp. are able to grow in culture. The location of each isolate, as well as the salinity (‰ or psu) used for the measurements, are also shown. n/a = data not available. A < or > indicates that the minimum or maximum temperature, respectively, that allows growth was not reached.

Species	Location of isolate	Salinity	Min	Max	Optimum	Reference
P. americana	Cape Fear River estuary, North Carolina, USA	26	15	> 32	25 ± 2.5	Miller & Kamykowski 1986
P. cuspidata	Nha Trang Bay, Vietnam	25–35	< 20	> 30	30	Doan-Nhu *et al.* 2008
P. granii	Ocean Station Papa, NE subarctic Pacific	n/a	< 8	20	~ 14	El-Sabaawi & Harrison 2006
P. multiseries	Pomquet Harbour, Nova Scotia, Canada	n/a	< 5	> 25	20–25	Lewis *et al.* 1993
	Chinhae Bay, South Korea	30	10	> 25	20	Cho *et al.* 2001
P. pseudodelicatissima[1]	Limfjord, Denmark	25	5	> 25	> 25	Lundholm *et al.* 1997
P. pungens	Chinhae Bay, South Korea	30	15	> 25	20	Cho *et al.* 2001
	Nha Trang Bay, Vietnam	26	< 26	> 26	26	Doan-Nhu *et al.* 2008

[1] May be multiple species within the *P. pseudodelicatissima* complex (*sensu* Lundholm *et al.* 2003; Amato & Montresor 2008).

less, salinity has been shown to be an important parameter, among several others, that correlates with the presence of *Pseudo-nitzschia* spp. Several studies have shown significant positive correlations between salinity and the appearance of species or groups of species, e.g. *P. delicatissima* (Caroppo *et al.* 2005) and the *delicatissima* group (Fehling *et al.* 2006; Sahraoui *et al.* 2009). As well, certain *Pseudo-nitzschia* spp. were associated with specific salinity ranges in the Chesapeake Bay (Thessen & Stoecker 2008), and stenohaline species were found in two different bays in Vietnam (Doan-Nhu *et al.* 2008).

Only one study (Doucette *et al.* 2008) has examined the effects of salinity on DA production. When acclimated to different salinities, the cell division rate of *P. multiseries* was greatest at the three highest salinities tested (20, 30 and 40 psu) but declined by about half at the lowest salinity (10 psu). Cellular DA and toxin production were also maximal at the highest salinities (30 and 40 psu). DA production declined significantly (three- to sevenfold) in cells adapted to lower salinities (10 and 20 psu). The authors suggested that *P. multiseries* was able to maintain a high growth rate at 20 psu, but at the expense of being able to produce DA at elevated levels. They hypothesized that energy generated from photosynthesis may have been diverted from DA production to the maintenance of an osmotic balance, required to survive at the low salinity. Their findings suggested that DA levels should be greatest in higher-salinity coastal waters compared with low-salinity estuaries, which was consistent with their field observations along the Louisiana coast. In spite of the above finding that DA production was greatest at the highest salinities, there is still no proof that DA is acting as an osmolyte (Bates 1998). There are also no further studies advancing how taurine (Jackson *et al.* 1992), sorbitol (Stewart *et al.* 1997) or other such compounds may play this role for *P. multiseries* growing at high salinities.

Irradiance

Earlier research on *P. multiseries* (reviewed by Bates 1998; Bates *et al.* 1998) showed that photosynthesis saturated at 100–600 µmol photons m^{-2} s^{-1} and growth at 80–200 µmol photons m^{-2} s^{-1}. The photosynthesis of *P. americana* saturated at 140 µmol photons m^{-2} s^{-1} (Miller & Kamykowski 1986). Growth rates of the oceanic *P. granii* increased when the irradiance was increased from 20 to 150 µmol photons m^{-2} s^{-1}, and photosystem II activity increased under the high irradiance (El-Sabaawi & Harrison 2006). The growth of *P. australis* saturated at 100 µmol photons m^{-2} s^{-1} (Cochlan *et al.* 2008). Surprisingly, there is still a paucity of studies on irradiance–growth–photosynthesis relationships for *Pseudo-nitzschia* spp.

Metabolic energy derived via photosynthesis is essential for DA production (Bates *et al.* 1991; Pan *et al.* 1998) and thus a minimum irradiance is necessary to satisfy this energy requirement. For *P. multiseries*, at least, this is 100 µmol photons m^{-2} s^{-1} or greater (reviewed by Bates 1998); this should also be determined for other species. Cultures of *P. australis* produced 24–130 times more DA when grown at 115 compared with 12 µmol photons m^{-2} s^{-1} (Cusack *et al.* 2002). DA is produced under continuous irradiance (e.g. Kudela *et al.* 2004b) as well as under different photoperiods, although comparisons have not been made to determine the optimum irradiance condition. However, Fehling *et al.* (2005) determined that cultures grown under a long photoperiod [18:6 light:dark (L:D)] had a higher division rate than those under a short photoperiod (9:15 L:D). As well, greater amounts of DA were produced under the long photoperiod, in support of the requirement for photosynthetic energy to produce DA; interestingly, more DA was released from the cells under the short photoperiod.

Pseudo-nitzschia multiseries cells are capable of surviving for up to 6 wk in darkness, whether or not they are given dissolved organic nitrogen in the form of glutamate plus glutamine (Mengelt & Prézelin 2002). This dark survival ability, however, does not necessarily provide them with a competitive advantage because other diatoms may survive for longer periods in darkness. Moreover, the time required to resume rapid growth upon return to the light was on the order of weeks. Thus, dark survival and upwelling of a seed population from the depths were deemed less likely mechanisms for initiating blooms. The heterotrophic ability of *Pseudo-nitzschia* spp., which would allow dark survival, has not yet been studied sufficiently (see below).

There is an apparent contradiction in the requirement for light in DA production. No DA was produced by *P. multiseries* during the dark cycle in a batch culture (Bates *et al.* 1991). In contrast, cellular DA did increase during the

Table 5. Summary of salinity data, showing the minimum (Min), maximum (Max) and the optimum salinity at which the *Pseudo-nitzschia* spp. are able to grow in culture. The location of each isolate and the temperatures (Temp) at which the salinity-growth experiments were carried out are also shown. A < or > indicates that the minimum or maximum salinity, respectively, that allows growth was not reached.

Species	Location of isolate	Temp (°C)	Min	Max	Optimum	Reference
P. americana	Cape Fear River estuary, North Carolina, USA	25	< 8	> 32	26	Miller & Kamykowski 1986
P. cuspidata	Nha Trang Bay, Vietnam	20	25	30	30	Doan-Nhu *et al.* 2008
	Nha Trang Bay, Vietnam	25	25	35	25–35	Doan-Nhu *et al.* 2008
	Nha Trang Bay, Vietnam	30	25	35	25–35	Doan-Nhu *et al.* 2008
P. delicatissima[1]	nearshore coastal Louisiana, USA	24.5	6.25	> 45	15–40	Thessen *et al.* 2005
	Terrebonne Bay, Louisiana, USA	24.5	6.25	> 45	10–30	Thessen *et al.* 2005
P. multiseries	Galveston, Texas, USA	20	21	> 34	25–28	Reap 1991
	Pomquet Harbour, Nova Scotia, Canada	15	15	> 48	30–45	Jackson *et al.* 1992
	Chinhae Bay, South Korea	20	20	> 50	20–40	Cho *et al.* 2001
	Santa Cruz, California, USA	19	10	40	15–40	Thessen *et al.* 2005
	Monterey Bay, California, USA	19	7	> 45	25–30	Thessen *et al.* 2005
	Monterey Bay, California, USA	19	10	40	20–40	Doucette *et al.* 2008
P. multistriata	Guanabara Bay, Brazil	22–23	< 15	> 40	25–40	Villac *et al.* 2004
P. pseudodelicatissima[2]	Limfjord, Denmark	25	12	> 35	25	Lundholm *et al.* 1997
	offshore coastal Louisiana	24.5	13	> 45	25–40	Thessen *et al.* 2005
	offshore coastal Louisiana	24.5	13	> 45	25–40	Thessen *et al.* 2005
	Bermuda	24.5	13	> 45	15–30	Thessen *et al.* 2005
P. pungens	Galveston, Texas, USA	20	21	> 34	25–28	Reap 1991
	Brudenell River, Prince Edward Island, Canada	15	< 6	30	15–30	Jackson *et al.* 1992
	Chinhae Bay, South Korea	20	10	> 50	20–30	Cho *et al.* 2001
	Guanabara Bay, Brazil	22–23	< 15	> 40	20–40	Villac *et al.* 2004
	Nha Trang Bay, Vietnam	26	20	35	25–35	Doan-Nhu *et al.* 2008

[1] May be multiple species within the *P. delicatissima* complex (*sensu* Lundholm *et al.* 2006; Quijano-Scheggia *et al.* 2009a, 2010).
[2] May be multiple species within the *P. pseudodelicatissima* complex (*sensu* Lundholm *et al.* 2003; Amato & Montresor 2008).

dark cycle when this species was grown in a silicon-limited chemostat culture (Bates 1998). Further research is required to resolve this.

Except for one study on UV irradiation (reviewed in Bates 1998), light quality has otherwise not been studied. Subsequent research has shown that UV-A exposure enhanced the primary production of *P. australis*, in contrast to the surrounding phytoplankton community in the Santa Barbara Channel (California, USA), perhaps via a photoprotective or photorepair mechanism (Gorga *et al.* 2002). UV-B exposure, however, inhibited primary production, as expected. Nevertheless, the enhancement by UV-A was still thought to provide a competitive advantage, especially at depths where UV-A and photosynthetically active radiation are high but UV-B is absent.

pH

The decline in ocean pH, due to atmospheric CO_2 increase or to respiration, as well as an increase in pH due to photosynthesis, affects phytoplankton by altering the inorganic carbon species available for uptake during photosynthesis (see below). *Pseudo-nitzschia multiseries* is capable of using both CO_2 and HCO_3^- (Trimborn *et al.* 2008), which gives it an advantage at both low and high pH. Despite the long-term ocean acidification accompanying global climate change, a more immediate interest is the high pH reached at the end of intense blooms or in batch cultures, which results in a decrease in CO_2 available for photosynthesis. When cultures reached a pH > 8.6, the division rate of *P. multiseries* slowed, then stopped (Lundholm *et al.* 2004). Because the cultures were not limited by light, vitamins, nitrate, silicate or phosphate, the authors argued that the pH specifically, and not inorganic carbon, was the factor that limited growth at stationary phase. DA was detected simultaneously with the rise in pH; thus they further argued that elevated pH, rather than silicate or phosphate limitation, was the trigger for DA production under their growth conditions.

Trimborn *et al.* (2008) found that *P. multiseries* cellular DA increased more than 70-fold with an increase in pH (i.e. 1.9 pg DA cell^{-1} at pH 7.9; 4.2 pg cell^{-1} at pH 8.4 and 140 pg cell^{-1} at pH 8.9; note that the latter cellular DA value is the highest ever reported for *P. multiseries*), thus supporting the results of Lundholm *et al.* (2004). Both papers speculated that the increased cellular DA may be due to a change in internal pH, thus favouring DA biosynthesis. If validated by others, this finding adds one more trigger for DA production, in addition to silicate and phosphate limitation and trace metals (see below). Contrary to the above studies, an earlier study showed no significant effect of pH on division rates of *P. multiseries* and *P. pungens* when grown at pH 5–9 (Cho *et al.* 2001). A recent study by Sun *et al.* (2011) also showed contrary results, i.e. an increase in cellular DA (from 2.0 to 10.0 pg cell^{-1}, i.e. fivefold) when the pH was decreased (from 8.38 to 7.95), in

this case because of addition of CO_2 (see below). Clearly, additional experiments are required to resolve these different findings about the effects of pH on growth, carbon limitation and DA production. This is also important because of the direct effect of pH on trace-metal availability (see below), one factor in bloom development (Hutchins *et al.* 1998).

NUTRITIONAL PARAMETERS AFFECTING GROWTH AND TOXICITY

Macronutrients

Nutrient enrichment of seawater has led to increases in *Pseudo-nitzschia* spp. abundance and may be responsible for an increase in harmful algal blooms (HABs) worldwide (Anderson *et al.* 2008; Heisler *et al.* 2008). Sometimes, however, links to eutrophication are not easily made, e.g. when there is a negative correlation between *Pseudo-nitzschia* abundance and ambient nutrient concentration (Dortch *et al.* 1997; Schnetzer *et al.* 2007). This may be caused by an immediate drawdown of nutrients by the developing bloom and may therefore obscure the true, longer-term relationship. When cells become nutrient limited they can no longer divide but are still able to photosynthesize and acquire energy. This energy may be used for the production of secondary metabolites, including DA (Pan *et al.* 1998).

SILICON AND PHOSPHORUS: Early studies showed that DA production by *P. multiseries* is triggered by silicate and phosphate limitation in cultures (Pan *et al.* 1996a, b, c; reviewed by Bates 1998). Later studies have confirmed this for silicate (Kudela *et al.* 2004b; Lundholm *et al.* 2004) and phosphate (Pan *et al.* 1996a; Hagström *et al.* 2010; Sun *et al.* 2011). DA production by *P. seriata* is similarly triggered (Fehling *et al.* 2004). Growth of *P. australis* in f/2 medium containing low concentrations of silicate also resulted in DA production (Cusack *et al.* 2002), as was also found for *P.* sp. cf. *pseudodelicatissima* (Pan *et al.* 2001), which could be identified as either *P. pseudodelicatissima* or *P. cuspidata* (Lundholm *et al.* 2003), and *P. cuspidata* (Trainer *et al.* 2009b). Studies are still required to determine if any other species are similarly triggered. Mathematical models have described the growth and DA production of *P. seriata* under limitations by silicate and phosphate (Davidson & Fehling 2006). It is possible, however, that other limiting factors (e.g. carbon, trace metals; see below) are responsible; these would still slow cell division, thus permitting DA to accumulate within the cells. The exact mechanism that triggers DA production nevertheless remains elusive.

Phosphorus limitation decreased the chlorophyll *a* content of *P. multiseries* (Pan *et al.* 1996a), which runs counter to an energy requirement for the observed increase in DA production. Silicon limitation, on the other hand, increased the chlorophyll *a* content of *P. multiseries* (Pan *et al.* 1996a) and decreased its efficiency of photosynthesis, as measured by variable fluorescence (Kudela *et al.* 2004b). In contrast to other diatoms, the lipid content of *P. multiseries* decreased, rather than increased, in response to silicon deficiency during stationary phase (Parrish *et al.* 1991). This was thought to be caused by light limitation in the dense cultures, but an alternative explanation is that shared precursors, such as acetyl coenzyme A, are channelled into DA rather than lipid synthesis at that time (Pan *et al.* 1998).

NITROGEN: In mesocosm experiments, species of the genus *Pseudo-nitzschia* responded to nitrate addition (Carter *et al.* 2005; Claquin *et al.* 2010). In the Gulf of Mexico, the abundance of wide *Pseudo-nitzschia* species ('*pungens*' group) and thin species ('*delicatissima*' group) has increased since the 1960s and 1980s, respectively (Parsons *et al.* 2002). A positive correlation between nitrate flow and *Pseudo-nitzschia* concentration suggested the Mississippi River as the nitrogen source. Nitrate from submarine groundwater discharge (as distinct from surface runoff or river discharge) is thought to have created a 'hot spot' for *Pseudo-nitzschia* spp. growth in coastal Alabama (USA) waters (Liefer *et al.* 2009; MacIntyre *et al.* 2011).

Nitrogen is indispensable to amino acid synthesis, thus to cell growth and division. As DA is an amino acid, it requires nitrogen to be synthesized. Early studies (Bates 1998; Pan *et al.* 1998), and later ones (Fehling *et al.* 2004; Kudela *et al.* 2004b) showed that N limitation resulted in diminished *P. multiseries* cell yield and no detectable DA production. However, some studies showed that low levels ($\sim$ 0.2 pg DA cell^{-1}) were produced (Kudela *et al.* 2004b; Calu *et al.* 2009). *Pseudo-nitzschia multiseries* can grow on multiple sources of nitrogen, both inorganic (nitrate, ammonium) (Thessen *et al.* 2009) and organic (urea, glutamine) (Hillebrand & Sommer 1996; Calu *et al.* 2009; Thessen *et al.* 2009), as can *P. delicatissima* (Ilyash *et al.* 2007, Loureiro *et al.* 2009b) and *P. australis* (Howard *et al.* 2007). *Pseudo-nitzschia delicatissima* took up ammonium more readily than urea but was able to grow on urea when other nitrogen sources were low, giving comparable photosynthetic rates with either substrate (Loureiro *et al.* 2009b). The growth rate of *P. delicatissima* was similar when nitrogen was provided as urea, nitrate or ammonium, or as an undefined form in high-molecular-weight organic matter. *Pseudo-nitzschia multiseries* is sensitive to high concentrations of ammonium, compared with *Skeletonema costatum*; four strains showed growth or photosynthetic impairment at concentrations $>$ 220 µM (Bates *et al.* 1993b). At the same time, DA production was enhanced two- to fourfold.

Interpretation of results about the ability of different species of *Pseudo-nitzschia* to take up and grow on different nitrogen sources must consider the finding that there is great interstrain variability, even if the strains are isolated from the same water sample. For example, of the five *P. fraudulenta* strains studied, two grew fastest on nitrate and ammonium, two on ammonium and one on urea (Thessen *et al.* 2009). One strain of *P. calliantha* grew fastest on nitrate and ammonium and the other on ammonium. The one commonality of all three species (*P. multiseries*, *P. fraudulenta* and *P. calliantha*) was that they all had higher growth rates on ammonium and lower growth rates on urea.

The effect of nitrogen source on DA production is just as unpredictable, with some strains of each species producing

more DA when grown with nitrate, ammonium or urea, and others showing no variation in toxicity with nitrogen source (Thessen *et al.* 2009). Cellular DA levels were highest in *P. multiseries* and only near the detection limit in toxic *P. fraudulenta* and *P. calliantha*. Intraspecific variation in toxin production was greater than the interspecific variation and could be caused by bacteria or genetics (or both).

The following studies included only one strain of a given species. *Pseudo-nitzschia australis* showed a preference for taking up nitrate, followed by glutamine, ammonium and urea (Cochlan *et al.* 2008). Growing *P. australis* with urea resulted in two times more DA than with nitrate and three times more than with ammonium or the control (Howard *et al.* 2007). The ability to produce DA when grown with glutamine was not tested. Growth of one strain of axenic *P. multiseries* with glutamate resulted in a higher cell number and 34 times more cellular DA than the axenic control, although this was still less than the xenic (containing bacteria) control (Lyons 2002); the results could not be replicated using another strain. Similar to *P. australis*, *P. multiseries* and *P. pungens* also produced more DA when grown with urea than with nitrate in batch and chemostat cultures (Calu *et al.* 2009). Curiously, the finding of cellular DA in *P. pungens* (up to 0.2 pg DA cell^{-1}), which would be the first report for a European strain, was not discussed.

Artificial sources of nitrogen must also be considered when carrying out experiments. Adding 2.1–8.2 mM Tris buffer, a primary amine, to cultures of *P. multiseries* (three different strains) enhanced DA production by two- to threefold, perhaps by providing a nitrogen source or acting indirectly on the cells, rather than by affecting pH (Douglas *et al.* 1993).

Taken together, these results indicate the ability of different *Pseudo-nitzschia* species to take advantage of the wide variety of nitrogen sources available in different environments. Of special concern is that urea enhanced the toxicity of *P. australis* (Howard *et al.* 2007) and *P. multiseries* (Calu *et al.* 2009), although more variable results were found for two other strains of *P. multiseries* and strains of *P. calliantha* and *P. fraudulenta* (Thessen *et al.* 2009). Nevertheless, the use of urea has increased threefold over the last 4 decades in certain agricultural areas of the world (Glibert *et al.* 2006), making this nutrient especially problematic.

ORGANIC NUTRIENTS AND HETEROTROPHIC ABILITY: Some organic sources of nitrogen were discussed above, but other studies have also suggested a heterotrophic ability for *Pseudo-nitzschia* spp. Addition of sewage effluent to microcosms resulted in the dominance of *P. multiseries* or *P. pungens* over other phytoplankton (Pan & Subba Rao 1997). After treating the effluent with UV light to break down organic material, *Pseudo-nitzschia* spp. no longer dominated, suggesting that members of this genus had been taking advantage of the presence of organic material for growth. Loureiro *et al.* (2009a) later demonstrated that addition of dissolved organic matter to seawater samples resulted in an increase in *Pseudo-nitzschia* spp., although not in chlorophyll *a*, suggesting that the cells fulfilled their nutritional needs via assimilation of the organic matter.

The heterotrophic capacity of *P. multiseries* has not been well studied. Acetate (labeled with ^{13}C or ^{14}C) was used in DA biosynthesis studies (Douglas *et al.* 1992; Ramsey *et al.* 1998) and has thus been indirectly shown to be taken up by the diatom. Although gluconic acid/gluconolactone apparently increased DA production in axenic cultures (see below), uptake of the compound was not demonstrated. The uptake of other organic substrates and their potential effects on DA production was investigated by Lyons (2002). No growth or DA production occurred when a strain of axenic *P. multiseries* was placed in darkness for 35 d in media supplemented with 28 mM glucose, acetate, gluconic acid/gluconolactone or glutamate, which argues against its ability for heterotrophy. However, other concentrations of these organic substrates should be tested, using several strains and different acclimation times, before coming to a solid conclusion about its heterotrophic ability.

Tang *et al.* (2010) recently determined that *P. pungens* requires cobalamin (B_{12}), biotin (vitamin B_7) and thiamin (vitamin B_1), whereas *P. multiseries* (two strains) requires only cobalamin (B_{12}), indicating different auxotrophic abilities. The significance of this difference is not yet known, especially with respect to the ability to produce DA.

CARBON: In most culture studies, with *P. multiseries* at least, nearly all of the DA is produced postexponential phase. At that time, the concentration of CO_2 is decreased because of photosynthetic uptake, the pH is high and the carbonate system is shifted toward higher proportions of bicarbonate and carbonate. Although *P. multiseries* is capable of using both of these forms of inorganic carbon (Trimborn *et al.* 2008), total inorganic carbon (TIC) becomes low. It is therefore possible that the available TIC concentration may modify DA biosynthesis during the stationary phase. This hypothesis was tested by amending medium f/2 (containing 1.9 mM TIC) with sodium bicarbonate to give 2.8 and 3.7 mM TIC (Bates & Léger 2006). These additions resulted in 4.8 times and 11.5 times more cellular DA, respectively, than the unamended control. The significant linear relationship between cellular DA concentration and initial TIC concentration suggested carbon limitation of DA biosynthesis. Likewise, agitation on a rotary shaker table as well as bubbling with air can increase DA production (unpublished results; Mafra 2009), another indication of possible inorganic carbon limitation. The bicarbonate addition experiments also showed a positive relationship between DA levels and pH, in support of Lundholm *et al.* (2004) and Trimborn *et al.* (2008) (see above).

Interestingly, results from a recent study showed that increasing the partial pressure (Pa) of CO_2 (pCO_2) in phosphorus limited *P. multiseries* semicontinuous cultures stimulated DA production 1.4-fold when supplemented with ~ 41 Pa [400 parts per million (ppm)] and fivefold with ~ 74 Pa (730 ppm) CO_2 (Sun *et al.* 2011). The differences in pH were small when CO_2 was added (8.38 to 7.94), but the greatest DA production occurred at the lowest pH, in contrast to Lundholm *et al.* (2004) and Trimborn *et al.* (2008). Surprisingly, even though the CO_2 addition increased the growth rate and DA production, carbon limitation was believed not to have been alleviated by the CO_2 addition (Sun *et al.* 2011). This is because the photosynthetic rates were lower in the phosphorus-limited

culture, which had the highest DA production, than in the phosphorus-replete culture, where DA production was lowest. As well, *P. multiseries* has a high affinity for CO_2 (Trimborn *et al.* 2008), which argued against the likelihood of inorganic carbon limitation. Nevertheless, results of Bates & Léger (2006) do support this possibility (see above). The ratio of carbon fixation to the degree of nutrient stress may be an important determinant of DA production (Sun *et al.* 2011). Clearly, additional research is required to verify the effects of pH and carbon limitation on toxin production and to understand mechanisms of action.

Trace-metal nutrients

LITHIUM: The first study conducted with trace metals and *Pseudo-nitzschia* concerned lithium, which was shown to stimulate DA production in *P. multiseries* (Subba Rao *et al.* 1998). Lithium was found at concentrations of 1.5 to 47.8 μM in Cardigan Bay, Prince Edward Island (Canada) during the DA episode of 1987–1988, presumably from waters flowing over an adjacent dump. Enrichment of a culture with 386 μM lithium, although substantially more than was found in the original episode, resulted in a higher cellular DA level (230 fg $cell^{-1}$ on day 17) than in the control (135 fg $cell^{-1}$ on day 25); released DA was also substantially higher. Because silicate and phosphate remained at nonlimiting concentrations, it was believed that lithium was responsible for enhancing DA biosynthesis, perhaps by increasing the levels of cyclic adenosine monophosphate (cf. Pan *et al.* 1998).

IRON AND COPPER: At least three situations can be described whereby toxic *Pseudo-nitzschia* blooms occur along the west coast of the United States in relation to differing concentrations of macronutrients, iron and copper. First, toxic blooms in the Juan de Fuca eddy (discussed above, Marchetti *et al.* 2004; Trainer *et al.* 2009a, b) occurred in waters that were not limited by silicate or phosphate. Instead, the lowest iron concentrations (< 0.5 nM) in the eddy were found where particulate DA was the highest (Trainer *et al.* 2009a), suggesting that iron limitation, not macronutrients, was responsible for triggering DA production. Second, toxic blooms occurred along the California coast where iron was not limiting, but neither were silicate and phosphate (Hutchins & Bruland 1998; Trainer *et al.* 2000; Kudela *et al.* 2004a). What then, could trigger DA production? Ladizinsky (2003) found high correlations between DA accumulation and elevated concentrations of copper from anthropogenic sources, suggesting that copper could have triggered DA production (see below). Third, areas of southern California experienced toxic blooms in the presence of low concentrations of macronutrients (Schnetzer *et al.* 2007; Seeyave *et al.* 2009), which may have been caused by the concurrent growth of the phytoplankton population; unfortunately, these studies did not report information about iron or copper.

The presence of both iron-replete and iron-limiting upwelling waters in California may be explained by the coastal bathymetry (Bruland *et al.* 2001). Those waters with a narrow continental shelf, thus depriving them of iron from sediments, and with no riverine iron inputs (e.g. Big Sur), can have limiting concentrations of iron similar to the mid-oceanic HNLC waters. Other upwelling areas (e.g. Peru) are also characterized by such 'coastal' HNLC waters (Eldridge *et al.* 2004). Iron bioavailability is further reduced in these waters by its complexation with strong organic ligands released by microbes (Wells *et al.* 2005). On the other hand, coasts with wide continental shelves and shallow bays with freshwater inputs (e.g. Monterey Bay) have high concentrations of iron that do not limit phytoplankton growth (Hutchins *et al.* 1998), although copper concentrations may also be high and potentially toxic (Ladizinsky 2003). The presence of toxigenic *Pseudo-nitzschia* spp. in waters that have both high and low iron and macronutrient concentrations indicates that there are multiple conditions, including macronutrients, iron and copper, that are conducive to these blooms. Given that macronutrients could not always explain the distribution of *Pseudo-nitzschia* spp. and the physiology of DA production, we now focus attention on the potential role of trace metals, particularly iron and copper, in this respect.

Interestingly, the addition of iron to mid-ocean HNLC waters that are limited by iron often results in the increased growth of pennate diatoms, including *Pseudo-nitzschia* spp. (as discussed above). Transition zones ('ecotones'), where iron-poor, nitrate-rich oceanic HNLC waters are transported toward iron-rich, nitrate-poor coastal waters, also promote the growth of *Pseudo-nitzschia* spp., as documented in the northeast Pacific Ocean (Ribalet *et al.* 2010). This indicates that low numbers of these species are always present in these waters, so they must have particular adaptations for low iron conditions. Furthermore, such seed populations are capable of responding rapidly to periodic inputs of iron from aeolian deposition or intermittent upwelling and mixing (Marchetti *et al.* 2006b; Silver *et al.* 2010).

Several strategies are used to survive at low ambient iron levels. In the case of pennate diatoms, oceanic *Pseudo-nitzschia* species have significantly lower iron cell quotas and higher iron-use efficiencies than the coastal *P. multiseries* (Marchetti *et al.* 2006a). Growth rates were therefore not as reduced when iron concentrations were low. The oceanic *P. granii* uses the iron-concentrating protein ferritin to enhance its storage of iron (Marchetti *et al.* 2009). This ability to sequester iron from sporadic inputs of this metal allowed *P. granii* to maintain near-maximum growth rates in culture, even at low iron concentrations. Although the coastal species *P. multiseries* also exhibits the ability to produce ferritin, it has only a twofold range in ferritin transcript abundance compared with a 20-fold range in *P. granii*. On the basis of ferritin sequence phylogenies, Marchetti *et al.* (2009) argue that this allowed *P. granii* to grow at low ambient iron concentrations and ultimately to radiate into open ocean waters.

Similar to other pennate diatoms (Leynaert *et al.* 2004), iron depletion in *Pseudo-nitzschia* spp. leads to a decrease in cell volume (by decreasing the cell width), cellular chlorophyll *a*, carbon, nitrogen and silicon cell quotas, maximum photochemical yield and growth rate (Marchetti & Harrison 2007). A decrease in cell size results in an increase in the cells' surface-to-volume ratio, which could increase the number of receptors available to scavenge the

Table 6. Comparison of domoic acid (DA) production by *Pseudo-nitzschia multiseries* growing in synthetic seawater with sufficient iron or with limited/depleted iron. n.d. = not determined.

Iron condition	Growth phase	Cellular DA (pg cell^{-1})	Dissolved DA production (fg cell^{-1} h^{-1})	Cellular DA production (fg cell^{-1} h^{-1})	Total DA production (fg cell^{-1} h^{-1})	Reference
Sufficient	stationary	40[1]	n.d.	n.d.	63.0	Bates *et al.* 2001
Limited		4[1]	n.d.	n.d.	4.7	
Sufficient	exponential	0.021	1.6	0.61	2.2	Maldonado *et al.*
Depleted		0.011	12.3	0.24	12.5	2002

[1] Includes dissolved DA.

limiting concentrations of iron (Eldridge *et al.* 2004). Iron-deficient *Pseudo-nitzschia* spp. cells were also characterized by elevated Si:N ratios, which increased twofold in the case of *P.* cf. *calliantha* (Marchetti & Harrison 2007).

A final strategy may involve DA as a chelator of iron and copper. The correlation between DA production and the presence of low concentrations of iron (Trainer *et al.* 2009a, b) or high concentrations of copper (Ladizinsky 2003) in field studies supports the hypothesis that one metabolic role of DA in *Pseudo-nitzschia* spp. may be to chelate iron and copper, for survival in low-iron or high-copper environments. This would be similar to the strategy of siderophore production by cyanobacteria during iron depletion or limitation to enhance their access to this metal (Wilhelm & Trick 1994), or metallothionein production to alleviate copper toxicity. There are some architectural similarities between the phytosiderophore mugenic acid and DA (Rue & Bruland 2001), which supports a chelation role of DA. Trace-metal chelation is possible because of the three carboxyl groups in the DA molecule (Bates *et al.* 2001). Values for the conditional stability constant of DA binding to iron and copper indicate that environmental concentrations of DA can compete for these trace metals with natural ligands in the seawater (Rue & Bruland 2001). The hypothesis was therefore put forward that DA production may be tied closely to the acquisition of iron or the detoxification of copper in marine waters.

A test of this hypothesis (Maldonado *et al.* 2002) showed that iron-deficient *P. multiseries* cells released DA more rapidly into the medium when grown with low levels of iron compared with iron-sufficient cells during the exponential phase of a semicontinuous culture (Table 6). Because 95% of the DA produced ended up in the medium, less DA remained within the iron-deficient cells than in the iron-sufficient cells. Nevertheless, the total DA production (intracellular DA plus extracellular release) was about six times faster for the iron-deficient than iron-sufficient cells (Table 6). Furthermore, addition of DA to the growth medium resulted in a threefold increase in iron uptake. When stressed by high copper levels, the cells released ~ 20 times more DA than the control. Similar results were found for *P. australis* producing DA (Ladizinsky 2003) and isodomoic C (Rhodes *et al.* 2004, 2006). It was thus argued that DA was produced and then released as a trace-metal chelator. This strategy would allow *Pseudo-nitzschia* cells to acquire iron at low concentrations by scavenging it, or to alleviate copper toxicity by chelation, which would reduce its bioavailability. Laboratory and field studies support these findings for copper (Ladizinsky 2003).

Further work carried out by some of the coauthors of the Maldonado *et al.* (2002) study extended these findings, but also revealed that the mechanisms could be much more complex (Wells *et al.* 2005). First, *P. multiseries*, *P. australis* and *P. fraudulenta* had low growth rates as well as low cellular DA concentrations only during an initial period of adaptation to iron-depleted media. As in Maldonado *et al.* (2002), this was interpreted to be caused by a greater release of DA from the cells to chelate the low levels of available iron. However, after acclimation, as shown by the resumption of a high growth rate (requiring about eight transfers), less DA was released because the cells were able to satisfy their iron requirement by accessing the DA-chelated iron; cellular DA then increased. Second, copper deficiency increased DA per cell by 20-fold, compared with a doubling when iron was the only limiting trace metal. Third, addition of dissolved DA increased growth rates in low-copper culture media, as well as in an iron-stressed Juan de Fuca eddy population composed mainly of *Pseudo-nitzschia* spp., to the same extent as did iron addition. Fourth, addition of ferrichrome, a nonmarine siderophore, to this field population to further increase iron limitation resulted in a three- to fourfold increase in cellular DA, but this was not observed when copper was also added. Taken together, these results were interpreted as showing a synergy between iron and copper with regard to controlling DA production. This led Wells *et al.* (2005) to develop a hypothesis that incorporated a role for DA and copper in alleviating iron limitation in *Pseudo-nitzschia* spp. It is based on the copper-dependent, high-affinity iron acquisition system of the yeast, *Saccharomyces cerevisiae*. They hypothesized that *Pseudo-nitzschia* cells actively release DA under low-iron conditions to first facilitate copper acquisition. This copper is then used to induce a high-affinity transport system that would allow it to access iron bound to strong ligands such as siderophores and therefore to alleviate the cells' iron deficiency. Wells *et al.* (2005) argue that without sufficient iron and copper, *Pseudo-nitzschia* will become more toxic by increasing its DA production rate to obtain the low levels of these trace metals via released DA. This hypothesis relies on the presence of a multicopper iron oxidase (Peers *et al.* 2005; Wells *et al.* 2005), as is found on the membrane of *S. cerevisiae* cells. The oxidase reoxidizes Fe(II) to Fe(III), making it available to the Fe(III) high-affinity transporters. It is found in the oceanic *Thalassiosira oceanica*, and a putative version of it was found in *Thalassiosira pseudonana* (Maldonado *et al.* 2006). Its presence, however, has not been verified in *P. multiseries*. Among other approaches, this will be deter-

mined when the whole-genome study of *P. multiseries*, now underway, is completed.

In contrast to the results of Maldonado *et al.* (2002) and Wells *et al.* (2005), which have been interpreted to mean that iron-stressed cells increase their DA production to alleviate iron limitation, Bates *et al.* (2001) had earlier found that total DA production by *P. multiseries* decreased, not increased, during iron limitation; i.e. the cellular DA levels and production rates were ~ 10-fold lower in iron-limited cells than in iron-sufficient cells during the stationary phase (Table 6). This decrease was attributed to a reduction in the iron-limited cells' ability to take up nitrogen and to produce sufficient photosynthetic energy, two processes that require iron and are also essential for DA production. It should be noted, however, that Bates *et al.* (2001) measured only total DA production (DA in the cells plus medium), and not dissolved DA, which means that direct comparisons cannot be made with the dissolved fraction in Maldonado *et al.* (2002). Nevertheless, differences in the results for total DA production can be compared and may perhaps be reconciled by noting that the cells used by Bates *et al.* (2001) were in stationary phase, whereas those used by Maldonado *et al.* (2002) were in exponential phase. Thus, the stationary-phase cells produced more DA whether or not they were iron limited, compared with the exponential-phase cells, as expected (Table 6). It could also be argued that the stationary-phase cells tested by Bates *et al.* (2001) were more iron stressed (= 'iron limited') than those used by Maldonado *et al.* (2002), which were in exponential growth and therefore less severely stressed (= 'iron depleted'). This could mean that the iron-depleted cells still had access to sufficient iron, via the hypothesized DA chelation mechanism, to function normally. In contrast, the stationary-phase, iron-limited cells may have depleted the available iron, thus impairing their ability to take up and metabolize nitrogen and to synthesize sufficient amounts of chlorophyll, and ultimately, of DA. The results suggest that DA production and release as a strategy to scavenge limiting concentrations of iron is useful only if the cells are mildly stressed by low concentrations of iron, and not if they are more severely iron limited. It then becomes important to know, e.g. for modeling or monitoring purposes, what state the cells are in within the spectrum of iron depletion to limitation, to predict if they will be capable of producing larger or smaller amounts of DA. This information may be gained, for example, by using variable fluorescence measurements (Bates *et al.* 2001; Kudela *et al.* 2004b). The finding of particulate DA at several depths in unamended HNLC waters at OSP (Trick *et al.* 2010) suggests that these waters were not severely iron limited.

The combined results of all previous studies with nutrients and trace metals suggest that DA would accumulate in the cells when triggered by silicate or phosphate limitation, but only when iron is still in excess. If iron also becomes limiting, then cellular DA levels would decrease because an increasing proportion of the cellular DA would be released by the cells. Maldonado *et al.* (2002) argue that this could account for the large variability in cellular DA levels observed during *Pseudo-nitzschia* blooms in coastal waters. It could also explain why little DA is produced by exponentially growing, nonlimited cells in batch cultures, whereas large amounts are produced during early to mid-stationary phase when limited by silicate or phosphate, but not by iron. In late-stationary phase, cellular DA decreases and increasing amounts of DA are released into the medium, perhaps triggered by iron limitation. To validate this hypothesis, measurements of DA production with respect to macronutrient and iron concentrations must be made during the entire growth cycle in batch culture, from exponential to late-stationary phase. It follows that increasing the concentration of iron, and possibly even of copper, in the growth medium may result in greater DA production and less release of DA from the cells.

Clearly, many physical factors are able to influence DA production, and others will likely be found. Ultimately, the only commonality among them may be how the factor affects gene expression within the DA biosynthetic pathway, which still requires fuller elucidation.

BIOLOGICAL PARAMETERS AFFECTING GROWTH AND TOXICITY

The appearance of *Pseudo-nitzschia* blooms is linked to numerous biotic factors, some of which are species specific. This makes it a challenge to understand bloom dynamics, which is essential to forecast bloom timing, intensity and location, and to predict cellular toxicity. Nevertheless, the growth of *Pseudo-nitzschia* with other phytoplankton and with bacteria may provide some clues.

Association with other phytoplankton

Most *Pseudo-nitzschia* spp. cells form chains that are free-living in the water column (e.g. Rines *et al.* 2002) or on the sediment surface (Dortch *et al.* 1997). Several examples, however, show *Pseudo-nitzschia* spp. living amongst colonies of other diatoms or other unicellular, photosynthetic algae: *P. pseudodelicatissima* in *Chaetoceros socialis* colonies (Gailhard *et al.* 2002; Rines *et al.* 2002); *P. americana* (Lundholm *et al.* 2002b; Nézan *et al.* 2007; Stonik *et al.* 2011) and *P. linea* (Lundholm *et al.* 2002b; Quijano-Scheggia *et al.* 2010) as single cells on *Chaetoceros* and *Odontella* spp. colonies; *P. delicatissima* as single cells in *Phaeocystis* colonies (Lundholm *et al.* 2002a; Sazhin *et al.* 2007); and *P.* cf. *granii* var. *curvata* in *Phaeocystis pouchetii* colonies (Sazhin *et al.* 2007). The advantages of these associations are unknown, but the colonies could provide a growth substrate or organic compounds, including protective aldehydes, that could benefit the colonies and the *Pseudo-nitzschia* cells. Note that the above identifications of *P. pseudodelicatissima* and *P. delicatissima* may in fact be any of the several new species named in the *P. pseudodelicatissima/cuspidata* complex (Lundholm *et al.* 2003) or the *P. delicatissima* complex (Lundholm *et al.* 2006), respectively (see above). Recently, the sexual pairing between parental cells of *P. australis* or *P. pungens* was observed on dense colonies of surf-zone diatoms (Holtermann *et al.* 2010). In that case, it is possible that the sexualized *Pseudo-nitzschia* cells (see above) could be taking advantage of the surf-zone diatom substrate to move toward each other.

Allelopathy

As with many other phytoplankton species (Granéli & Hansen 2006), it is reasonable to assume that *Pseudo-nitzschia* may have allelopathic effects on sympatric species. However, thus far, no studies have been able to demonstrate allelopathic interactions between *Pseudo-nitzschia* and any other algal species. Early trials showed no influence of DA addition on diatom (*C. gracilis*, *Skeletonema costatum*) growth (Windust 1992). Subba Rao *et al.* (1995) reported an allelopathic interaction between toxic *P. multiseries* and the diatom *Rhizosolenia alata* in a mixed culture. However, the results show that increasing the proportion of *R. alata*, or its filtrate, resulted in a decrease in *P. multiseries* growth, not the other way around. Thus, it was not proven that *P. multiseries* produced an allelopathic compound. Likewise, Lundholm *et al.* (2005b) later confirmed that DA addition had no effect on the growth of nine different phytoplankton species, nor did toxic *P. multiseries* have an effect on four algal species in mixed-batch culture experiments.

A recent meta-analysis of possible allelopathic effects on HAB species (but not *Pseudo-nitzschia*) found that, in the few studies that included low cell numbers, none detected any allelopathic effects (Jonsson *et al.* 2009). Modelling showed that the concentrations of such compounds in the bulk seawater would be too low to be effective. They therefore doubted that allelopathy is a mechanism in HAB formation. Nevertheless, their modelling also showed an order-of-magnitude-higher concentration of a putative allelopathic compound immediately around the cell, which opens the possibility of such effects during cell-to-cell encounters. For example, Olson & Lessard (2010) argued that the localized high DA concentrations within the diffusion zone of a cell, in contrast to its concentration in the bulk seawater, could play a role as a microzooplankton grazing deterrent (but see below). Likewise, the proximity of the cells within thin layers may facilitate the usefulness of any allelopathic compound or increase the likelihood of cell-to-cell encounter, leading to sexual reproduction.

Bacteria

Amongst other biotic factors is the existence of bacteria, which have a complex relationship with HAB species. This includes both positive and negative effects on algal growth, as well as a role in phycotoxin production (reviewed by Kodama *et al.* 2006). Bacteria are capable of lysing some diatoms, although there is no solid proof that bacteria have killed *Pseudo-nitzschia* spp. There is only the observation that bacteria attached to *P.* sp. cf. *pseudodelicatissima* appeared to coincide with the lysis of the diatom cells (Pan *et al.* 2001). As well, empty frustules of *P. multiseries* in late stationary phase were packed with bacteria, but this may have occurred after cell death (Kaczmarska *et al.* 2005a). Otherwise, positive effects have been documented. The presence of bacteria in *P. multiseries* cultures increased the cells' growth rate and longevity relative to axenic cultures (Douglas *et al.* 1993), although another study found that axenic and xenic *P. multiseries* cultures had the same growth rates (Kobayashi *et al.* 2009).

Bacteria isolated from *P. multiseries* (Kaczmarska *et al.* 2005a) and *P. pungens* (Sapp *et al.* 2007) cultures were found to be members of the Alphaproteobacteria, Gammaproteobacteria and Bacteroidetes. Another study, using automated ribosomal intergenic spacer analysis (ARISA) profiling, reported Flavobacteria, Alphaproteobacteria and Gammaproteobacteria in both *P. multiseries* and *P. multiseries* (Guannel *et al.* 2011). More specifically, *Alteromonas*, *Moraxella* and *Spirosoma*-like bacteria were reported in other *P. multiseries* cultures (Stewart *et al.* 1997). All bacteria found are Gram negative (Bates *et al.* 1995a).

Depending on the study, the bacterial composition in *Pseudo-nitzschia* cultures shifted, or not, over time in culture. A shift in the bacterial community within a *P. pungens* culture was observed 4–12 mo after isolating the cells (Sapp *et al.* 2007). Moreover, the bacterial assemblage in the freshly isolated culture was different from that in the waters from which the diatoms were originally isolated. In contrast, Guannel *et al.* (2011), using ARISA profiling, found that two strains of nontoxic *P. delicatissima* did not exhibit any shifts in bacterial assemblages over 9–14 mo in culture, nor did toxic *P. multiseries* and *P.* sp. 233 over a 2-mo period. Moreover, some of the assemblages in the cultures were similar to those found in field samples where the diatoms originated, again, contrary to Sapp *et al.* (2007). Interestingly, Guannel *et al.* (2011) found, using ARISA profiling, no differences in bacterial community composition between the exponential and stationary phases for all *Pseudo-nitzschia* strains tested, including the five toxic strains. As well, the composition of the attached and free-living communities (defined operationally, on the basis of what passed through or was retained on filters) did not differ significantly for 9 of the 11 *Pseudo-nitzschia* strains assessed. These findings argue against the hypothesis that bacterial composition is in part responsible for the decline in toxicity in culture (see above) or for the differences in toxicity between exponential-phase and stationary-phase cultures. However, their study did report differences between toxigenic and nontoxigenic *Pseudo-nitzschia* species. Toxigenic strains hosted fewer bacterial ARISA operational taxonomic units, in comparison with nontoxigenic strains. Although both *P. multiseries* (toxigenic) and *P. delicatissima* (nontoxigenic) cultures hosted members of the Gammaproteobacteria and Flavobacteria, 16S rDNA sequencing revealed that the specific bacteria coexisting with each *Pseudo-nitzschia* strain differed on a finer taxonomic scale, i.e. *P. multiseries* hosted members of the order *Oceanospirillales*, whereas *P. delicatissima* hosted *Alteromonadales*. The results of Guannel *et al.* (2011) provided evidence for their hypothesis that DA plays a role in structuring bacterial community composition, i.e. that the growth environment may become enriched with DA-utilizing, DA-tolerant or DA-intolerant bacteria in response to exposure to DA.

The existence of intracellular bacteria in *P. multiseries* is debated, with some authors not finding any (Bates *et al.* 1995b) and others reporting molecular evidence of them (Kobayashi *et al.* 2003). In the latter case, Kobayashi *et al.* (2003) argued that the intracellular bacteria originated from the culture medium, although it was not clear how the *P. multiseries* cells acquired them. Extracellular bacteria may

be either epiphytic or free living. Pan *et al.* (2001) reported an increase in bacterial abundance during the stationary phase of *P.* sp. cf. *pseudodelicatissima*, with the majority of them attached to the cell surface. In *P. multiseries* cultures, only ~ 40% of cells carried epiphytic bacteria (one to five per cell), but their number and the diversity of morphotypes increased during growth in batch culture (Kaczmarska *et al.* 2005a), in contrast to the above ARISA results (Guannel *et al.* 2011). Two subclones of *P. multiseries* from the same parent stock, but grown under slightly different conditions in a different laboratory, developed different epiphytic bacterial assemblages (Kaczmarska *et al.* 2005a).

The presence of bacteria in *Pseudo-nitzschia* cultures has raised considerable interest because several research groups have independently demonstrated that axenic cultures produce less DA than xenic cultures, and that bacteria can enhance DA production (Douglas *et al.* 1993; Bates *et al.* 1995a, b; Kotaki *et al.* 1999; Kobayashi *et al.* 2003). Not all bacteria enhanced DA production, but a wide variety from different locations was capable of it. Axenic cultures produced 2- to 95-fold less DA than xenic cultures, and readdition of the bacteria restored DA production (Douglas *et al.* 1993; Bates *et al.* 1995a, b). There is still no proof that bacteria themselves are able to produce DA (Bates *et al.* 2004). DA production by *N. navis-varingica* was also enhanced by bacteria (Kotaki *et al.* 2000), and bacteria were suggested to play a role in the isodomoic acid composition in this species (Kotaki *et al.* 2008). The effect of epiphytic bacteria, specifically, on DA production has yet to be determined, although the *P. multiseries* strain that had the greatest number and diversity of epiphytic bacteria also had the highest DA production (Kaczmarska *et al.* 2005a). Notwithstanding the above results of Guannel *et al.* (2011), the effects of free-living and attached bacteria, their abundance and composition must be considered, along with the abiotic factors discussed above, in studies of toxin production.

It is still not known how the bacteria enhance DA production. One hypothesis is that some bacteria have an 'antagonistic relationship' with *Pseudo-nitzschia* cells, which then respond by producing DA (Kaczmarska *et al.* 2005a). That DA may, however, be beneficial or not to the *Pseudo-nitzschia* cells (Guannel *et al.* 2011). Another hypothesis involves the production of gluconic acid/gluconolactone from glucose by certain bacteria, including *Alteromonas* sp. (Osada & Stewart 1997). This compound is a 'powerful sequestering agent' that can tie up nutrients, and may therefore be a competitor of DA, also a chelating agent (although for trace metals). Adding gluconic acid/gluconolactone to an axenic culture of *P. multiseries* enhanced DA production in a dose-dependent manner (Osada & Stewart 1997; Stewart 2008). It was argued that DA could be produced by the diatom as an external chemical scavenger to counter nutrient stress during the stationary phase (Stewart 2008). However, it was not specified which nutrients the gluconic acid/gluconolactone scavenged, whereas there is evidence (see above) that DA is a chelator for copper and iron (Maldonado *et al.* 2002). Furthermore, when gluconic acid/gluconolactone was added to a xenic culture, DA production was not enhanced, but rather decreased; no explanation could be found for this incongruity (Stewart 2008). Thus, the gluconic acid/gluconolactone hypothesis requires further verification.

Another hypothesis is that the bacteria may be supplying nitrogenous compounds or other precursors that are used directly in DA production, or indirectly as 'elicitors' of toxin production (Bates 1998). Three of four bacteria isolated from a toxic *P. multiseries* culture produced *N*-acyl homoserine lactones (N-AHLs), a group of chemical signal molecules used in quorum sensing (Johnston *et al.* 2001). The authors postulated that N-AHLs may be a possible route by which bacteria influence diatom toxin production. Adding bacterial extracts to axenic cultures did not affect DA production, suggesting that a dynamic interaction is required between the diatom and bacterial cells (Bates 1998). This is supported by Kobayashi *et al.* (2009), who suspended an axenic *P. multiseries* culture, contained within a cellophane tube, into a xenic culture. They determined that the cellular DA level of the cells within the tube was much lower than that of the cells outside the tube. This led them to conclude that direct contact between living bacteria and *P. multiseries* cells is necessary for producing high levels of DA in this diatom species. Clearly, bacteria are playing an important but complex role in toxin production, but the details of this are still elusive.

Viruses and fungi

Viruses are known to infect diatoms, and these are mainly species specific (e.g. Nagasaki *et al.* 2005). To date, viruses have only been hypothesized to infect *Pseudo-nitzschia* spp., although research is underway to study this (Carlson *et al.* 2009). Recently, parasitic oomycetes (Figs 5, 6) and chytrids were found to infect *P. pungens* in Canada and elsewhere (Hanic *et al.* 2009). Their role in controlling *Pseudo-nitzschia* bloom dynamics and toxicity should be considered.

IMPACTS OF DOMOIC ACID

Since 1987, numerous observations have been made about how DA-producing blooms affect shellfish, fish, seabirds and marine mammals (Table 7). For example, blue mussels (*Mytilus edulis*) and clams (*Mya arenaria*) from the Bay of Fundy (Canada) became contaminated during July to October 1988, after filtrating toxic *P. pseudodelicatissima* (Martin *et al.* 1990) [the identity of this diatom may be *P. calliantha*, but this is still in dispute (Lundholm *et al.* 2003)]. High DA concentrations are often observed in crabs and mussels from the US west coast (Horner *et al.* 1997). In contrast to most molluscan shellfish (see below), other animals are negatively affected. From 1989 to 1991, hundreds of brown pelicans (*Pelecanus occidentalis*) and Brandt's cormorants (*Phalacrocorax penicillatus*) died in Monterey Bay (California) after ingesting contaminated anchovies (Work *et al.* 1993). Seabirds were also affected in 1996 in Mexico after ingesting anchovies and sardines contaminated by toxic *Pseudo-nitzschia australis* (Sierra-Beltrán *et al.* 1997). The DA contamination of seabirds is perhaps better known than people think. Indeed, the Alfred Hitchcock film "The Birds" (1963) may have been inspired

from a real event of birds becoming violent with humans after consuming fish contaminated with DA, near Santa Cruz, California (Dybas 2004).

Marine mammals are also heavily affected. In 1998, > 400 sea lions (*Zalophus californianus*) that had fed on contaminated anchovies died in California (Scholin *et al.* 2000; Howard *et al.* 2007; reviewed by Bargu *et al.* 2011a, b). Surviving sea lions exhibited neurological dysfunction, including head waving, ataxia and abnormal behavior, similar to that shown by DA-intoxicated mice. Identical syndromes were previously observed in sea lions and sea otters in 1978, 1986, 1988 and 1992 (Scholin *et al.* 2000). The cause of sea lion deaths and strandings is complex, involving a combination of environmental and physiological factors in addition to toxic *Pseudo-nitzschia* blooms (Bargu *et al.* 2010). DA later caused the death of minke whales (*Balaenoptera acutorostrata*) in Southern California (Fire *et al.* 2010), and of pygmy and dwarf sperm whales (*Kogia* spp.) in southeastern and mid-Atlantic US waters (Fire *et al.* 2009). It also contaminated North Atlantic right whales (*Eubalaena glacialis*) in the Bay of Fundy (Leandro *et al.* 2010a); a copepod (*Calanus finmarchicus*) was the potential vector (Leandro *et al.* 2010b).

DA is accumulated in the digestive gland and tissues of filter-feeders as a result of their feeding on toxic *Pseudo-nitzschia* cells. There is no evidence that DA may be accumulated directly from the seawater. The mechanics and ability of molluscan shellfish and zooplankton to filter-feed on DA-producing *Pseudo-nitzschia* cells have been studied by few researchers. Detailed information is available only for oysters (*Crassostrea virginica*) and mussels (*M. edulis*) feeding on *P. multiseries* (Mafra *et al.* 2009a, b, 2010). Oysters filtered fewer cells when fed with *P. multiseries* as the only food source, compared with a mixture of *P. multiseries* and nontoxic algae, and produced pseudofeces when a threshold concentration of *Pseudo-nitzschia* was reached. There was a selective rejection of *Pseudo-nitzschia* cells into pseudofeces when these cells were mixed with flagellates, but not when mixed with other diatoms. Rejection can also occur on the gills. As a consequence of these selective mechanisms, oysters accumulate lower concentrations of DA than do other shellfish, including mussels (Mafra *et al.* 2009a). Interestingly, the presence of DA played no role in the oysters' selection process; rather, other intrinsic properties of diatoms in general were believed to be responsible. A similar conclusion was reached for microzooplankton feeding on *Pseudo-nitzschia* cells: the presence of DA or toxic *Pseudo-nitzschia* cells had no effect on microzooplankton grazing or growth (Olson & Lessard 2010). Likewise, this study showed that a diatom-consuming dinoflagellate did not feed on toxic or nontoxic *Pseudo-nitzschia* cells, indicating the involvement of factors other than DA. These, and previous studies (e.g. Lincoln *et al.* 2001; Tester *et al.* 2001), indicate that DA *per se* plays no role as a grazing deterrent, in contrast to an earlier hypothesis (Bates *et al.* 1989).

With the exception of Pacific oysters (*Crassostrea gigas*) (Jones *et al.* 1995a, b), bivalves are very resistant to DA, even at concentrations as high as 790 μg g^{-1}, as seen in healthy mussels of the original 1987 ASP event (Bates *et al.* 1989). As with DA-resistant Pacific razor clams (*Siliqua patula*), they may have proteins that sequester DA or mute the receptors, thus limiting DA fixation (Trainer & Bill 2004). Shellfish do not have a nervous system as developed as that in birds and mammals, which may explain the differences in susceptibility. DA affects the behavior of anchovies (Lefebvre *et al.* 2001), krill (Bargu *et al.* 2006), sea lions (Goldstein *et al.* 2008; Bargu *et al.* 2011b), and northern fur seals (*Callorhinus ursinus*) (Lefebvre *et al.* 2010) via nervous system dysfunction. DA also has genotoxic effects on fish, such as the Nile tilapia (*Oreochromis niloticus*) (Cavas & Konen 2008). The leopard shark (*Triakis semifasciata*) is so far the only vertebrate unaffected by DA (Schaffer *et al.* 2006). These sharks posses the molecular targets for DA, but are resistant to it when injected with high doses.

DA can also accumulate to high concentrations in other benthic organisms during toxic *Pseudo-nitzschia* blooms (Kvitek *et al.* 2008). This may be another source of contamination for predators like seabirds, sea lions and fish. Contaminated fish have been caught and consumed by recreational anglers in southern California, posing an additional risk to humans (Vigilant & Silver 2007; Mazzillo *et al.* 2010). Consumption of contaminated menhaden (*Brevoortia patronus*) is yet another potential vector of DA to humans (Del Rio *et al.* 2010).

The effects of long-term consumption of DA by humans and animals are unknown, although evidence is slowly being gathered (Pulido 2008; Lefebvre & Robertson 2010). Low, chronic doses of DA given to rats (Truelove *et al.* 1996) or monkeys (Truelove *et al.* 1997) did not induce clinical or histopathology abnormalities. However, chronic, sublethal exposure of DA to sea lions led to epilepsy (Ramsdell & Stafstrom 2009). Moreover, prenatal exposure of rats to DA at mid-gestation caused learning and memory impairments that persisted into adulthood (Levin *et al.* 2006). DA can cross the placenta, accumulating in the amniotic fluid and entering the brain tissue of prenatal rats (Maucher & Ramsdell 2007). A 5-yr prospective epidemiological cohort study of American Indians has been initiated to determine if the DA levels in razor clams in the Pacific Northwest of the United States are placing them at risk of illness (Grattan *et al.* 2009).

Some countries/locations, such as Japan (Kotaki *et al.* 1999), Australia (Takahashi *et al.* 2007), Chile (Suárez-Isla *et al.* 2002), Chesapeake Bay (Thessen & Stoecker 2008), the Gulf of Naples (Orsini *et al.* 2002; Cerino *et al.* 2005) and the Gulf of Mexico (Liefer *et al.* 2009; Thessen *et al.* 2010; MacIntyre *et al.* 2011), have detected DA produced by toxic species of *Pseudo-nitzschia*; however, there have so far been no resulting serious ecosystem or human-health-related consequences. This may be because toxic cell numbers (Thessen & Stoecker 2008) or cellular DA concentrations (Orsini *et al.* 2002; Cerino *et al.* 2005) are too low, the species present are not suitable for being grazed upon (Thessen *et al.* 2010) or conditions are not conducive to DA production (see above). Indeed, not all strains of the same *Pseudo-nitzschia* species are toxic (Bates *et al.* 1998; Bates 2000), and other strains produce DA in variable amounts (Kudela *et al.* 2004b). This variable toxicity within a species may be explained by genetic factors, instrumentation that is not sensitive enough to detect the DA, misidentification of the species or use of growth conditions that do not trigger DA production.

Table 7. Chronology of domoic acid contamination involving various animals used for human consumption or not ([1]).

Location	Year	Affected animal		*Pseudo-nitzschia* species implicated	Reference
		Animal group	Species		
Prince Edward Island, Canada	1987	shellfish	*Mytilus edulis*	*P. multiseries*	Bates *et al.* 1989
Prince Edward Island, Canada	1988 to 1989	shellfish	*Mytilus edulis*	*P. multiseries* or *P. pungens*	Bates *et al.* 1998
Bay of Fundy, Canada	1988	shellfish	*Mya arenaria*, *Mytilus edulis*, *Volsella modiolus*, *Placopecten magellanicus*	*P. pseudodelicatissima* or *P. calliantha*	Martin *et al.* 1990
California, USA	1989 to 1991	fish	*Poeobius meseres*	*P. australis*	Buck *et al.* 1992
Washington & Oregon coasts, USA	1991	shellfish, crabs	*Siliqua patula*, *Cancer magister*	*P. australis*?	Horner & Postel 1993
California, USA	1991 to 1992	fish	*Engraulis mordax*	*P. australis*	McGinness *et al.* 1995
California, USA	1991	birds	*Pelecanus occidentalis*,[1] *Phalacrocorax penicillatus*[1]	*P. australis*	Fritz *et al.* 1992; Work *et al.* 1993
California, USA	1991 to 1993	shellfish	*Siliqua patula*	unknown	Wekell *et al.* 1994
Washington, USA	1992	shellfish	*Mytilus edulis*, *Crassostrea gigas*	unknown	Horner & Postel 1993
Denmark	1992	shellfish	*Mya arenaria*, *Mytulis edulis*	*P. pseudodelicatissima*	Lundholm & Skov 1993
New Zealand	1993 to 1996	shellfish	not specified	*P. australis*	Rhodes 1996
Baja California peninsula, Mexico	1996	birds, fish	*Pelecanus occidentals*,[1] *Scomber japonicus*	unknown	Sierra-Beltrán *et al.* 1997
Portugal	1997 to 2000	shellfish	*Mytilus edulis*, *Cerastoderma edule*, *Scrobicularia plana*, *Venerupis pullastra*, *Ostrea edulis*, *Ensis* spp., *Ruditapes decussata*	*P. australis*-like	Vale & Sampayo 2001
Atlantic coast, USA	1997 to 2005	marine mammals	*Kogia breviceps*,[1] *K. Sima*[1]	not directly linked	Fire *et al.* 2009
California, USA	1998	fish, marine mammals	*Engraulis mordax*, *Zalophus californianus*[1]	*P. australis*	Lefebvre *et al.* 1999
Washington, USA	1998	shellfish	*Siliqua patula*	*P. pseudodelicatissima*	Adams *et al.* 2000
California, USA	1998	marine mammals	*Zalophus californianus*[1]	*P. australis*	Scholin *et al.* 2000
California, USA	1998 to 2006	marine mammals	*Zalophus californianus*[1]	unknown	Bejarano *et al.* 2008a
Scotland	1999	shellfish	*Pecten maximus*	*P. australis*	Campbell *et al.* 2001
Ireland	1999	shellfish	*Pecten maximus*, *Mytilus edulis*, *Crassostrea edulis*, *Ensis siliqua*	*P. australis*	James *et al.* 2005
Brittany, France	1999	shellfish	*Donax trunculus*	*P. multiseries*	REPHY
California, USA	1999 to 2000	shellfish, crabs	*Mytilus edulis*, *Emerita analoga*		Ferdin *et al.* 2002
France	1999 to 2000	shellfish	*Mytilus galloprovincialis*, *Ruditapes decussatus*, *Donax trunculus*	*P. pseudodelicatissima* or *P. multiseries*	Amzil *et al.* 2001
California, USA	2000	fish	*Citharichthys sardidus*, *Scomber japonicus*, *Thunnas alalunga*, *Eopsetta jordani*, *Atherinopsis califoniensis, Hyperprosopon argenteum*, *Engraulis mordax*, *Sardinops saga*	*P. australis*	Lefebvre *et al.* 2002a, b
California, USA	2000	krill	*Euphausia pacifica*[1]	*P. australis*	Bargu *et al.* 2002b
California, USA	2000	squid	*Loligo opalescens*[1]	*P. australis*	Bargu *et al.* 2008
France	2000	shellfish	*Donax trunculus*	unknown	Thébaud *et al.* 2005
Prince Edward Island, Canada	2000	shellfish	*Mytilus edulis*	*P. multiseries*	Bates & Richard 2000
Argentina	2000	fish, shellfish	*Mytilus edulis*, *Engraulis anchoita*	*P. australis*	Negri *et al.* 2004
California, USA	2000 to 2001	benthic species	*Emerita analoga*, *Urechis caupo*, *Citharichthys sordidus*, *Nassarius fossatus*, *Pagurus samuelis*, *Neotrypaea californiensis*, *Dendraster excentricus*, *Olivella biplicata*[1]	*P. australis*	Kvitek *et al.* 2008
Portugal	2000 to 2001	fish, shellfish	*Sardina pilchardus*, *Engraulis mordax*, *Mytilus edulis*, *Sardinops sagax*, *Cerastoderma edule*, *Venerupis pullastra*, *Ruditapes decussate, Crassostrea japonica, Ensis* spp., *Solen* spp.	unknown	Costa & Garrido 2004

Table 7. Continued

Location	Year	Affected animal		*Pseudo-nitzschia* species implicated	Reference
		Animal group	Species		
California, USA	2001	fish	*Genyonemus lineatus*, *Leptocottus armatus*	*P. australis*	Fire & Silver 2005
California, USA	2002	marine mammals	*Zalophus californianus*,[1] *Delphinus capensis*,[1] *Delphinus delphis*[1]	unknown	de la Riva *et al.* 2009
Monterey Bay, California, USA	2002	squid	*Loligo opalescens*[1]	*P. australis*	Bargu *et al.* 2008
France	2002	shellfish	*Donax trunculus*	unknown	Thébaud *et al.* 2005
Portugal	2002	crab	*Polybius henslowii*	not directly linked	Costa *et al.* 2003
California, USA	2002 to 2003	fish	*Citharichthys sordidus*, *Eopsetta exilis*, *Eopsetta jordan*, *Psettichthys melanostictus*, *Errex zachirus*, *Microstomus pacificus*, *Pleuronectes vetulus*, *Pleuronicthys decurrens*, *Hippoglossus stenolepis*	*P. australis*	Vigilant & Silver 2007
Portugal	2002 to 2003	fish	*Sardina pilchardus*	*P. australis*	Costa & Garrido 2004
Greece	2002 to 2003	shellfish	*Mytilus galloprovincialis*, *Venus verucosa*	unknown	Kaniou-Grigoriadou *et al.* 2005
Ireland	2003	shellfish	*Pecten maximus*	unknown	Bogan *et al.* 2007
Washington, USA	2003	shellfish	*Mytilus edulis*	*P. australis*	Bill *et al.* 2004
Portugal	2003 to 2004	cephalopods, fish	*Octopus vulgaris*, *Sepia officinalis*, *Eledone moschata*, *Eledone cirrhosa*	not directly linked	Costa *et al.* 2004, 2005a, b
California, USA	2003 to 2004	crabs, fish	*Pleuroncodes planipes, Scomber japonicus, Trachurus symmetricus*, *Citharichthys sordidus*, *Zaniolepis latipinnis*	*P. multiseries*	Busse *et al.* 2006
Isle of Man	2003 to 2004	shellfish	*Pecten maximus*	unknown	Bogan *et al.* 2007
California, USA	2003 to 2004	fish	*Errex zachirus*, *Microstomus pacificus*, *Pleuronectes vetulus, Pleuronectes decurrens*, *Genyonemus fineatus*, *Gymnocanthus tricupsis*	*P. australis*-like	Trainer *et al.* 2008
Florida, USA	2004	marine mammals	*Tursiops truncatus*[1]	unknown	NMFS 2004
Australia	2004	shellfish	*Saccostrea glomerata*, *Modiolus proclivis*, *Donax deltoides*	unknown	Takahashi *et al.* 2007
Mexico	2004	fish, marine mammals, birds	*Delphinus capensis*,[1] *D. delphis*,[1] *Zalophalus californianus*,[1] *Pelecanus occidentalis*,[1] *Sardinops* spp.	unknown	Sierra-Beltrán *et al.* 2005
Chile	2004	shellfish, tunicate	*Mytilus chilensis*, *Aulacomya ater*, *Protothaca thaca*, *Pyura chilensis*[1]	*P. australis*	López-Rivera *et al.* 2009
West coast, France	2004	shellfish	*Pecten maximus*	*P. australis* or *P. multiseries*	Nézan *et al.* 2010
Vietnam	2004 to 2005	shellfish	*Spondylus versicolor*, *Spondylus cruentus*	unknown	Ha *et al.* 2006; Dao *et al.* 2009
Denmark	2005	shellfish	*Mytilus edulis*	*P. seriata*	Lundholm *et al.* 2005a
Bay of Fundy, Canada	2005 to 2006	marine mammal	*Eubalaena glacialis*[1]	*P. seriata*, *P. cuspidata*, *P. delicatissima*	Leandro *et al.* 2010a
California, USA	2005 to 2009	marine mammal	*Callorhinus ursinus*[1]	*Pseudo-nitzschia* spp.	Lefebvre *et al.* 2010
Japan & Thailand	2006	shellfish	*Spondylus* spp.	unknown	Takata *et al.* 2009
Korea	2006 to 2007	shellfish	*Mactra veneriformis*, *Peronidia venulosa*	unknown	Choi *et al.* 2009
Croatia	2006 to 2008	shellfish	*Mytilus galloprovincialis*, *Ostrea edulis*, *Pecten jacobaeus*, *Flexopecten proteus*	unknown	Ujević *et al.* 2010
California, USA	2007	marine mammal	*Balaenoptera acutorostrata*[1]	*P. australis*	Fire *et al.* 2010
Angola	2007	shellfish	*Dosinia orbignyi*, *Venerupis corrugata*, *Mactra glabrata*	unknown	Blanco *et al.* 2010
West coast, France	2007	shellfish	*Pecten maximus*	unknown	REPHY
Louisiana, USA	2007 to 2008	fish	*Brevoortia patronus*	unknown	Del Rio *et al.* 2010
Scotland	2008 to 2009	marine mammal	*Phoca vitulina*[1]	unknown	Hall & Frame 2010
West coast, France	2010	shellfish	*Pecten maximus*	*P. australis*	Nézan *et al.* 2010

GAPS IN KNOWLEDGE

The genus *Pseudo-nitzschia* has been the main subject of numerous studies since its discovery as a DA producer in 1988, yet there is still much to learn, analyze and understand. Some of the gaps in knowledge previously identified (Bates 1998) have been at least partially filled: 'triggers of DA production, other than silicon and phosphorus limitation'; 'role of trace metals (especially iron) in mediating DA production and *Pseudo-nitzschia* growth'; 'details of the life cycle of *Pseudo-nitzschia* species' (although not in relation to DA production); 'physiological studies of *Pseudo-nitzschia* species other than *P. multiseries*'; and 'identification of other producers of DA'. However, the remaining gaps, along with some new ones identified below, still remain. Knowledge gaps specific to life-cycle events are outlined in Mann & Bates (2001).

Only a few papers have investigated, specifically, the intraspecific variability in *Pseudo-nitzschia* physiology, including toxin production (e.g. Bates *et al.* 1999; Kudela *et al.* 2004b; Thessen *et al.* 2009; Amato *et al.* 2010) and association with bacteria (Guannel *et al.* 2011). However, because of the magnitude of the differences among strains, future studies should consider multiple strains to arrive at more solid conclusions and generalities about a given species. Molecular markers appropriate for detecting intraspecific variability, including use of microsatellite analysis, could be applied to these culture studies, in addition to their current application in field studies. They could also be relevant for investigating the progeny obtained during mating studies.

Physiological and ecological roles of DA production

After 23 years of research, the role of DA is still not known with certainty, other than its possible advantages for *Pseudo-nitzschia* in chelating iron and copper (Wells *et al.* 2005); these finding, however, require corroboration by other laboratories. Some of the roles hypothesized by Bates (1998) have been addressed, but require more study. For example, the 'osmolyte hypothesis', whereby DA could serve as an osmolyte in response to increasing salinity, has not been fully addressed. Although DA production was greatest at elevated salinities (Doucette *et al.* 2008), there was no proof that DA was acting as an osmolyte. The 'antifeedant hypothesis', whereby DA could act as a grazing deterrent, is not supported by the more recent research (Lincoln *et al.* 2001; Tester *et al.* 2001; Mafra *et al.* 2009a; Olson & Lessard 2010). Likewise, the 'allelopathy hypothesis', whereby DA could be deleterious to other phytoplankton, is not supported by evidence (Lundholm *et al.* 2005b). The 'excretion hypothesis', whereby DA may be produced and released as a mechanism to get rid of 'excess' photosynthetic energy when cells are no longer able to grow optimally, remains to be addressed.

Presence/absence of a resting stage

Centric diatoms form resting spores, which are very different from vegetative cells in appearance and physiology. Some pennate diatoms form resting stages, which have undergone physiological and cytoplasmic changes but remain morphologically similar to the vegetative cells of the species. In either case, these forms enable the cells to survive during unfavourable conditions (McQuoid & Hobson 1996). Amato *et al.* (2005) hypothesized that *Pseudo-nitzschia* spp. may have a 'quiescent phase' during which cell growth is reduced. However, there is contradictory information regarding the existence of resting stages in *Pseudo-nitzschia* spp. McQuoid & Godhe (2004) indicated that *Pseudo-nitzschia* spp. are not known to form a resting stage, and none was found in sediments. On the other hand, Orlova & Morozova (2009) provide evidence of *Pseudo-nitzschia* sp. 'resting cells' in recent sediments of Peter the Great Bay (eastern Russia). Knowing if such a resting stage exists is important for understanding bloom formation and disappearance. From where do cells that initiate new blooms originate, and what is their physiological condition? A resting stage could enhance the survival of *Pseudo-nitzschia* from one growth season to another, regardless of the conditions. It could also decrease the number of cell divisions over a year, allowing them to survive for a longer period without undergoing sexual reproduction.

Genomics of sexual stages

When cells are undergoing sexual reproduction, it is quite easy to identify the different stages in cultures (Davidovich & Bates 1998). However, this is more difficult in field samples, where *Pseudo-nitzschia* spp. sexual events have only been observed twice (Holtermann *et al.* 2010; Sarno *et al.* 2010). In natural seawater, sexual stages represent only a low percentage of total cell numbers (Mann 1988; Sarno *et al.* 2010), plus they are a challenge to collect, as they might be destroyed during sampling. This makes it difficult to identify the sexual stages and to carry out experiments on them. Therefore, little is known about these stages and their genomics. Sexual reproduction allows genetic recombination between strains, but it is not known how sexual reproduction is regulated, how it might be related to DA production, what determines the production of 'male' or 'female' cells (i.e. what the genes are that determine mating types), how cells of opposite mating type find each other (involvement of pheromones?) and how genetic recombination occurs within the *Pseudo-nitzschia* genus. This information would help to understand the genetic structure of *Pseudo-nitzschia* populations, how new species appear and why different strains of the same species can produce, or not, DA. One way to identify the sexual stages and cells of opposite mating type is to develop genetic probes against them (Mann & Bates 2001).

Whole genome of *Pseudo-nitzschia* spp. and complete DA biosynthetic pathway

Although numerous studies on *Pseudo-nitzschia* biology have been published over the last few years, the genome of *Pseudo-nitzschia* spp. still remains incomplete. Comparisons between the genomes of toxigenic and apparently nontoxic *Pseudo-nitzschia* species are also lacking. Such studies are required to determine why different strains of the same species are not always toxic. The whole-genome

sequencing of *P. multiseries* is still ongoing (Parker *et al.* 2009). When completed, this will help to understand *Pseudo-nitzschia* physiology and how cells adapt to such a broad range of environmental parameters. It will also provide more information about which genes are involved in DA production (Boissonneault 2004). This will allow the development of molecular probes to identify which species at least have the genes for DA production, and when and why the cells become toxic. Furthermore, sequencing the whole genome may help to better assess the role of DA. Nevertheless, knowing the whole genome of *Pseudo-nitzschia* is not enough; the biosynthetic pathway of DA is still incompletely known (Pan *et al.* 1998; Thessen 2007). The regulation of the enzymes involved in this pathway remains unresolved and the relationship between DA biosynthesis and the cell division cycle (Bates 1998) requires clarification. Moreover, it is still not known where DA is stored within cells. The new molecular tools being developed (summarized by Kudela *et al.* 2010) should help to answer some of these questions.

Mechanisms of DA production and decline

DA production is modulated by factors other than various environmental parameters. Indeed, the amount of DA produced by *P. multiseries* decreases with the age of the culture, as does cell size. Is there a causal relationship between cell volume and the ability to produce DA? Otherwise, no one has been able to explain this decrease in toxicity. The mechanisms leading to an increase in DA production, when triggered by silicon or phosphorus limitation and iron deficiency/iron excess, remain unknown and are likely different from each other. Do these limitations induce DA production to contend with an unfavourable environment, or is it an indirect consequence of these limitations? Is there a synergy between macro- and micronutrient limitation in modulating DA production? Knowledge of the complete biosynthetic pathway and of the whole genome would help to analyze gene expression under these limitations in relation to DA production.

Biological control of DA production and of bloom dynamics

Although *Pseudo-nitzschia* spp. do not appear to produce allelopathic compounds, other phytoplankton species may still affect DA production. As well, competition for resources or grazing by predators may be other factors that modulate DA production. Of the biotic factors, only bacteria have been studied so far and these have been shown to enhance DA production. However, there is still incomplete knowledge about which groups of bacteria may be more conducive to enhancing DA production, and about how closely associated the bacteria must be to achieve this. The hypothesis concerning the bacterial production of gluconic acid/gluconolactone, as a chelator that prompts *Pseudo-nitzschia* to produce DA to counter this competition (Stewart *et al.* 1997), requires validation. Finally, studies are needed to determine how bacteria boost DA production.

The role of viruses in controlling *Pseudo-nitzschia* bloom dynamics or modifying cell physiology to trigger DA production is just in its early stage of study (Carlson *et al.* 2009). Only one study has been published that describes oomycete and chytrid fungal parasite infections of *P. pungens*, although the phenomenon is known to be more widespread (Hanic *et al.* 2009). It is not known which parasite species are responsible, or how many other *Pseudo-nitzschia* species may be affected. The potential importance of fungal parasites in controlling *Pseudo-nitzschia* bloom dynamics and toxin production is unknown. Sequencing the genes of parasites would help to identify their life stages and to quantify their prevalence and infection rates. These tasks are so far hampered by the inability to grow the fungal–*Pseudo-nitzschia* pair in culture.

Heterotrophic ability

Conflicting or incomplete evidence, presented above (Pan & Subba Rao 1997; Lyons 2002; Mengelt & Prézelin 2002), indicates that more research is required before solid conclusions can be reached regarding the heterotrophic ability of *Pseudo-nitzschia* spp. Factors to be considered when carrying out such experiments include: the concentration of each organic substrate, the pH of the medium after addition of the organics, preincubation for different times with several substrates to possibly induce uptake and use of both axenic and xenic controls. As well, care must be taken to avoid bacterial contamination in the treatments containing the organics. Tests are required for photoheterotrophy (or photo-organotrophy; ability to use organic molecules as the only carbon source and solar radiation as the energy source), carried out in the light, and for chemoheterotrophy (or chemo-organotrophy; ability to use organic compounds both as a source of carbon and as a source of energy), carried out in darkness.

Resolution of conflicting findings

Several conflicting findings require resolution, including the ability of toxigenic *Pseudo-nitzschia* spp. to produce DA in darkness (Bates *et al.* 1991; Bates 1998), the finding of different responses to nitrogen when different strains of the same *Pseudo-nitzschia* species are studied (Lyons 2002; Thessen *et al.* 2009) and the effects of pH on DA production (Lundholm *et al.* 2004; Trimborn *et al.* 2008; Sun *et al.* 2011), including its mechanisms of action.

Ability to predict blooms reliably

These gaps in knowledge concerning the biology of *Pseudo-nitzschia* and its production of DA make it difficult to predict blooms and their toxicity reliably, especially at a local scale. Recent models of *Pseudo-nitzschia* bloom dynamics (Anderson *et al.* 2009; Lane *et al.* 2009; Palma *et al.* 2010) are a step in the right direction, but more exact data and additional parameters are required to improve their accuracy. In particular, trace metals, biotic factors and species composition (or even the morphology-based groups) have yet to be incorporated. Results of further experimental work and field sampling will slowly fill in these gaps, resulting in more reliable predictive models at different temporal and geographic scales.

SUMMARY

Recent scientific breakthroughs have revealed new information about *Pseudo-nitzschia* species identification, molecular biology, physiology, toxicity and distribution. Molecular methods are now used regularly to examine phylogenetic relationships among *Pseudo-nitzschia* species and to assist in species identification in field samples and cultures; this has allowed the discovery of new species, some of which are cryptic or pseudo-cryptic. From 1993 to 2011, 17 new species have been described; 15 of these are since 2002. When possible, these identifications have been supported by mating studies, but this approach is only recent. Fourteen *Pseudo-nitzschia* spp. have now been shown to be toxigenic in culture.

Although most of the attention still remains focused on *P. multiseries*, and marginally on *P. pungens, P. seriata* or *P. australis*, other coastal and oceanic species have recently been included in experimental studies: *P. pseudodelicatissima*, *P. delicatissima*, *P. brasiliana*, *P. multistriata*, *P. calliantha*, *P. cuspidata*, *P. granii* and *P. fraudulenta*. The diversity of species studied gives a broader view of the differences between species, including coastal and oceanic, regarding toxicity and factors controlling DA production.

New triggers or enhancers of DA production have recently been found, in addition to the already known triggers caused by silicon or phosphorus limitation. Iron deficiency or copper excess are believed to enhance DA production and release from the cells, because of the ability of DA to chelate these trace metals. Chelation would render low concentrations of iron more bioavailable to the cells, or protect the cells from potentially toxic, high concentrations of copper. High salinities (30–40 psu) enhanced DA production by *P. multiseries*, the only species studied so far. The role of inorganic carbon, whose concentrations are controlled by CO_2 addition or removal, remains unclear because of conflicting evidence. For example, DA production was reported to be enhanced by both high and low pH, and was also shown to be limited, or not, by TIC concentration.

Organic sources of nitrogen, i.e. glutamine and urea, were shown to enhance DA production, relative to inorganic nitrogen sources, by several species of *Pseudo-nitzschia*, although there are many inter- and intraspecies variations. Such information will affect the design of field studies, as well as decisions about which nutrients are measured and which nutrient parameters are included in models. The importance of a relationship between iron and copper, as well as between macro- and micronutrients, was also revealed. Additional complexities in the interactions of bacteria with *Pseudo-nitzschia* cells in relation to enhancing DA production were discovered. Laboratory studies should include more than one strain of *Pseudo-nitzschia* because of intraspecies variations in the response to environmental factors affecting DA production.

An increasing number of field studies have been conducted to find links between the occurrence, abundance, succession and toxicity of *Pseudo-nitzschia* spp. and environmental parameters at a local scale. These have demonstrated the challenge in determining the trigger for DA production (i.e. silicon, phosphorus or iron deficiency, copper excess, presence of organic nitrogen), which may vary depending on location. Experiments and field sampling have shown that artificial or natural fertilization of HNLC regions of open oceans with iron selects for *Pseudo-nitzschia* spp., including at least two toxigenic species (*P. turgidula* and *P.* cf. *granii*). This is the first time that DA production has been demonstrated for species growing in open ocean waters.

Contamination of diverse animal genera by DA has been documented worldwide and has led to the closures of shellfish-harvesting areas. All trophic levels, from zooplankton to marine mammals and birds, accumulate DA, although its consequences on ecosystem function have been only partially elucidated.

A major advance has been the knowledge gained about the sexual reproduction of *Pseudo-nitzschia* species. First demonstrated in the laboratory in 1998 with *P. multiseries* and *P. pseudodelicatissima* (which may be *P. calliantha*), it has now been documented in 14 *Pseudo-nitzschia* species. This advancement has allowed the production of new large cells for further laboratory study and the confirmation that strains are members of the same species. Only rudimentary information is available about its implications for the toxicity of cells and its significance for bloom dynamics, factors important for incorporating into mathematical models. Recently, sexual reproduction was observed for the first time in the field. Further advances will be gained by studying the molecular biology of sexual stages.

ACKNOWLEDGEMENTS

Two reviewers provided constructive comments. We thank the following for current information about *Pseudo-nitzschia* spp. distribution: Inna Stonik (Russia), Lesley Rhodes (New Zealand) and Gustaff Hallegraeff (Australia). Anne Thessen and Louis Hanic provided additional information.

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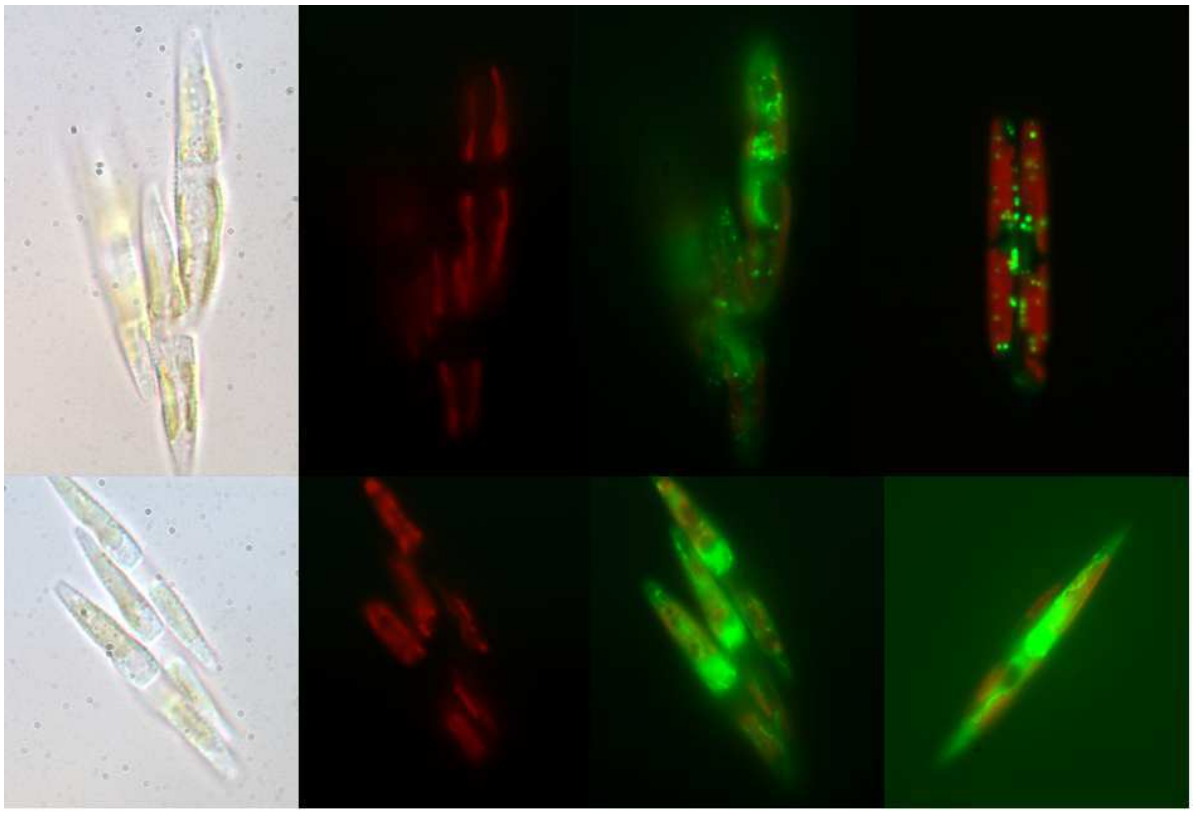
Received 25 March 2011; accepted 29 July 2011
Associate editor: Philip Orr

COMMENT SUIVRE LES VARIATIONS PHYSIOLOGIQUES DE *PSEUDO-NITZSCHIA* SPP. ? 2



Pseudo-nitzschia multiseries et *P. delicatissima*, marquées au BODIPY (en haut) et au FDA (en bas).

Article :

Lelong A., Hégaret H., Soudant P. 2011. Cell-based measurements to assess physiological status of *Pseudo-nitzschia multiseries*, a toxic diatom. *Research in microbiology* 162:969-981.

2.1. Préambule

Depuis une trentaine d'années, les scientifiques ont commencé à s'intéresser à l'écophysiologie du phytoplancton (par exemple Bates, 1998). Les premières études consistaient uniquement à suivre le taux de croissance des algues dans différents environnements contrôlés (par exemple Rueter et al., 1981). Sont ensuite apparues les mesures de chlorophylle *a* en environnement contrôlé ou naturel, avec au départ un simple dosage global, puis ensuite le dosage des différents pigments et enfin des mesures de photosynthèse (par exemple Appenroth et al., 2001). Une microalgue ne se résume cependant pas uniquement à ses chloroplastes, la photosynthèse seule ne permettant pas de comprendre de quelle façon les cellules sont, ou non, affectées par leurs conditions environnementales. Depuis quelques années, différents outils, issus pour la majorité de la médecine, ont été adaptés pour permettre des mesures plus précises de la physiologie des cellules, dont notamment la cytométrie en flux (par exemple Jochem, 2000).

L'objectif de ce chapitre est de présenter (i) les espèces sur lesquelles cette thèse a été menée et leurs conditions de culture (partie 2.2.) et (ii) la mise au point des mesures de la physiologie des cellules d'une espèce de *Pseudo-nitzschia*, *P. multiseries*, à l'aide de nouveaux outils (cytométrie en flux et "pulse-amplitude modulated" fluorimetry, ou PAM), pour certains jamais encore appliqués au milieu marin (partie 2.3., Lelong et al., 2011). Ces outils permettent de quantifier l'efficacité de la photosynthèse et d'estimer le fonctionnement du métabolisme primaire des cellules, mais aussi le stockage d'énergie excédentaire sous forme de lipides, la mortalité des cellules et des bactéries libres dans le milieu. Le but de ces mesures est de mieux comprendre et caractériser le fonctionnement de la cellule et de savoir à quel niveau le métabolisme est affecté dans des conditions environnementales variables. Ces mesures ont été appliquées au suivi d'une culture tout au long de sa croissance. Comme l'espèce étudiée est une espèce productrice d'acide domoïque, la quantité d'acide domoïque excrétée et cellulaire a également été mesurée.

2.2. Espèces modèles

Les études ont été menées sur deux espèces de *Pseudo-nitzschia* : *Pseudo-nitzschia multiseries* (Hasle) Hasle et *Pseudo-nitzschia delicatissima* (Cleve) Heiden (Figure 1).

Fig. 1: Cellules de *Pseudo-nitzschia delicatissima* Pd08RB et *Pseudo-nitzschia multiseries* CLNN-16 observées en lumière blanche, objectif 100.

Deux souches différentes de *P. multiseries* ont été utilisées durant cette thèse : CCAP 1061/32 (isolée en Angleterre) et CLNN-16 (isolée dans la baie de Fundy, Canada). La souche CCAP 1061/32 a été identifiée par Véronique Creach tandis que la souche CLNN-16 m'a été gracieusement donnée après identification par N. Lewis. La souche de *P. delicatissima* utilisée a été isolée en Rade de Brest par Béatriz Beker au printemps 2008 et nommée Pd08RB. Son identification a été réalisée premièrement par microscopie (de façon à déterminer son groupe) puis par biologie moléculaire. Après extraction de l'ADN de cette souche, le fragment ITS-1 a été amplifié puis séquencé au laboratoire de Milford (Connecticut, USA), lors d'un séjour de 2 mois que j'ai effectué en 2009. Le fragment ainsi séquencé a été aligné sur les séquences existantes de *Pseudo-nitzschia* dans GenBank et identifié comme appartenant à l'espèce *P. delicatissima*.

Ces deux espèces ont été cultivées en milieu f/2, dans des armoires de culture permettant un contrôle de la température (16°C) et de la lumière (130 µmol photons m^{-2} s^{-1}, cycle jour/nuit de 12:12h). Dans ces conditions, les deux souches de *P. multiseries* produisaient de l'acide domoïque à des niveaux détectables par kit ELISA (kit Biosense), contrairement à *P. delicatissima*. Ces deux espèces ont été maintenues dans les mêmes conditions de culture tout au long de ma thèse, conditions qui ont été les mêmes pour tous les articles présentés dans ma thèse, à l'exception de l'article traitant de la toxicité en cuivre (cultures à 17°C et 155 µmol photons m^{-2} s^{-1}) et des limitations en fer et cuivre (milieu de culture AQUIL).

2.3. Article 2 – Cell based measurements to assess physiological status of Pseudo-nitzschia multiseries*, a toxic diatom*

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Research in Microbiology 162 (2011) 969–981

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Cell-based measurements to assess physiological status of *Pseudo-nitzschia multiseries*, a toxic diatom

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Received 5 January 2011; accepted 9 May 2011
Available online 13 June 2011

Abstract

Diatoms of the genus *Pseudo-nitzschia* are potentially toxic microalgae, whose blooms can trigger amnesic shellfish poisoning. The purpose of this study was to test and adapt different probes and procedures in order to assess the physiological status of *Pseudo-nitzschia multiseries* at the cell level using flow cytometry. To perform these analyses, probes and procedures were first optimized for concentration and incubation time. The percentage of dead *Pseudo-nitzschia* cells, the metabolic activity of live cells and their intracellular lipid content were then measured following a complete growth cycle. In addition, chlorophyll autofluorescence and efficiency of photosynthesis (quantum yield) were monitored. The concentration and viability of bacteria present in the medium were also assessed. Domoic acid (DA) was quantified as well. Just before the exponential phase, cells exhibited high metabolic activity, but low DA content. DA content per cell became most abundant at the beginning of the exponential phase when lipid storage was high, which provided a metabolic energy source, and when they were surrounded by a high number of bacteria (high bacteria/*P. multiseries* ratio). These physiological measurements tended to decrease during exponential phase and until stationary phase, at which time *P. multiseries* cells did not contain any DA nor store any lipids, and started to die.
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Keywords: Flow cytometry; Cell physiology; Domoic acid; *Pseudo-nitzschia multiseries*; Bacteria; Fluorescent probes

1. Introduction

Pseudo-nitzschia is a potentially toxic diatom genus with a worldwide distribution. Some species are able to produce domoic acid (DA), an amnesic shellfish toxin leading to food poisoning (Sierra-Beltrán et al., 1998) with a few cases of mortality reported in humans (Wright et al., 1989), and hundreds of cases of sea bird (Sierra-Beltrán et al., 1997; Work et al., 1993) and marine mammal mortality (Scholin et al., 2000; Fire et al., 2009; de la Riva et al., 2009). These poisonings often occurred following a bloom of *Pseudo-nitzschia* spp. The reasons why these blooms occurred are poorly known. Some studies tried to create models to predict their occurrence (Anderson et al., 2009; Lane et al., 2009), but the determinism of each bloom seems different. Although factors enhancing or decreasing *Pseudo-nitzschia* cell toxicity have been intensively studied, they remain unclear. The study of *Pseudo-nitzschia* spp. physiology may help to understand why a bloom appears and becomes toxic. Tools to assess the physiological status of microalgae are still fairly rare. Photosynthetic capacities of *Pseudo-nitzschia* spp. have been studied under different conditions (Ilyash et al., 2007; El-Sabaawi and Harrison, 2006), but do not provide enough information to assess cell physiological status. In addition to photosynthetic parameters and chlorophyll content, other parameters have sometimes been studied in diatoms, e.g. silicification (Leblanc et al., 2005; Kroger and Poulsen, 2008) and carbohydrate levels (De Philippis et al., 2002; Magaletti et al., 2004), but these are also insufficient to characterize physiological processes occurring inside the cell. It is therefore important to develop and

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doi:10.1016/j.resmic.2011.06.005

then simultaneously measure several different physiological parameters that may help to better understand the factors or status associated with toxin production.

Assessment of cell physiology using fluorescent probes is a well-known subject in medicine (Greenspan et al., 1985; Knot et al., 2005). Among the numerous fluorescent probes available to assess cell physiology, some can be adapted to cultures of unicellular organisms. They allow measurements of different physiological parameters such as metabolic activity (with fluorescein diacetate, FDA), intracellular lipid content (Nile red (NR) and BODIPY), total DNA (SYBR Green) and mortality (SYTOX Green). Some of these probes have been used in microalgal studies for several years but are often limited to microscopic observations or spectrofluorimetric methods (Dempster and Sommerfeld, 1998; Okochi et al., 1999). The latter allow measurement of an entire population, but differences between cells cannot be observed. Microscopic observations allow cell-by-cell analyses, but are time-consuming, while fluorescence quantification is difficult. On the other hand, flow cytometry (FCM) allows rapid analysis of the morphological and fluorescence characteristics of unicellular organisms and individual cells. Although FCM has a long history of routine use in medical analyses, the first experiments using FCM on microalgae were run only about thirty years ago (Olson et al., 1983; Yentsch et al., 1983) and the approach remains only a minor component for measuring the physiology of phytoplankton. Some probes have already been tested on microalgae using FCM, such as FDA (Dorsey et al., 1989; Brookes et al., 2000; Jochem, 1999), SYTOX Green (Veldhuis et al., 1997) ad SYBR Green (Marie et al., 1997). Each of these probes provides new insights into understanding how cells react under different conditions, e.g. dark adaptation (Jochem, 1999), but they have never been applied simultaneously to assess physiological status in a more comprehensive manner.

This study sought to assess the physiological status of *P. multiseries* using a set of cell-based measurements. To attain this objective, different measurements were developed and adapted to this species: (i) to better understand its physiology under culture conditions, and (ii) to elucidate the relationship between production of DA and cell physiological status. The morphofunctional characteristics of *P. multiseries* cells were assessed by FCM using different fluorescent probes (FDA, BODIPY 493/503, NR, SYTOX Green, SYBR Green and propidium iodide) and measurement of chlorophyll autofluorescence. Quantum yield (QY), which is a measurement of the efficiency of photosynthesis, was measured using a pulse amplitude-modulated (PAM) fluorometer. Dissolved and particulate DA were measured on each culture using an ELISA assay. DA is a secondary metabolite, presumed to be produced when cells have more energy than necessary for primary metabolism. Thus, primary metabolism was assessed using FDA and esterase activity. The availability of energy was assessed by measuring storage lipids, as extra energy is stored by microalgae under a lipid form. The concentration of bacteria may influence DA production by *P. multiseries*, as they are known to enhance DA production (Bates et al., 1995). Chlorophyll and QY measurements enabling determining whether culture is healthy and were completed by measurement of the dead cell percentage.

2. Materials and methods

2.1. Cultures

Strain CCAP 1061/32 of *P. multiseries* (isolated in 2007 in England) was used for the experiments. Cultures ($n = 6$) were grown in sterilized f/2 medium (Guillard and Hargraves, 1993) at 15.6 °C (±0.2 °C) and 131 ± 16 µmol photons m^{-2} s^{-1} (light:dark photoperiod of 12:12 h). Seawater used for f/2 medium was first filtered at 0.22 µm to eliminate any remaining bacteria (which was confirmed by flow cytometric measurements, as described below) and then autoclaved. Cultures were xenic and grown without antibiotics. Before each sampling, cultures were homogenized by gentle manual stirring. Almost all cells were present as single cells in our cultures; sometimes, cells formed 2 cell chains. For flow cytometry analysis, they were all considered as single cells.

2.2. Physiological measurements

Measurements were made with a FACScalibur flow cytometer (BD Biosciences, San Jose, CA USA) using an argon blue laser (488 nm). Three fluorescence signals could be detected by the flow cytometer: FL1 (green, 530 nm), FL2 (orange, 585 nm) and FL3 (red, 670 nm). Red fluorescence was linearly linked to the chlorophyll content of the cells and was used as a discriminating characteristic to detect the microalgae (Fig. 1). Bacteria were detected on the FL1 channel (Fig. 2), with different settings from those used for microalgae analysis. Cell counts were estimated from the flow-rate measurement of the flow cytometer (Marie et al., 1999), as all samples were run for 45 s. The flow rate from the FCM was controlled every two days. Forward scatter (FSC, light scattered less than 10°) and side scatter (SSC, light scattered at a 90° angle) were also measured. FSC is commonly related to cell size and SSC to cell complexity. The same instrument settings were used for the entire duration of the experiment to allow comparison between days.

2.2.1. Bacteria

Quantification of free-living bacteria in the *P. multiseries* culture and the percentage of dead bacteria in the culture were assessed by adding SYBR Green I (Molecular probes, Invitrogen, Eugene, OR, USA) at a final concentration of 1/10,000 of the commercial solution and propidium iodide (PI, Sigma, St. Louis, MO, USA) at 10 µg ml^{-1} to each sample. During analyses, aggregates of bacteria were taken into account with correction according to aggregate size (Fig. 2). Bacterial counts were estimated as described for *Pseudo-nitzschia* cells, using FL1 as a discriminating characteristic (due to SYBR green fluorescence staining).

2.2.2. Mortality

To assess *Pseudo-nitzschia* cell mortality, we used a cell membrane-impermeable dye, SYTOX Green (Molecular

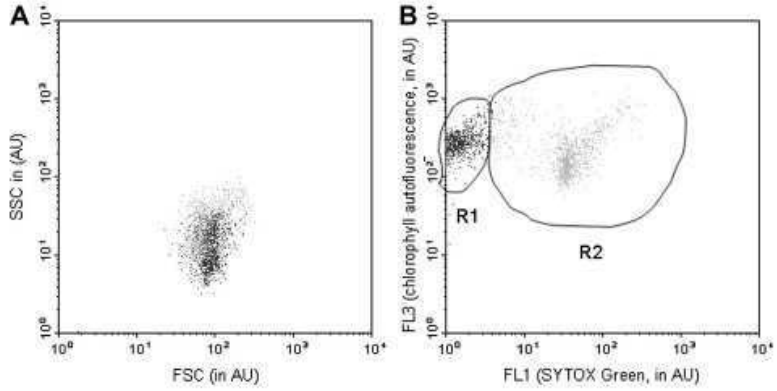

Fig. 1. Cytograms of 50/50 dead/live cells of *P. multiseries* stained with SYTOX Green. A. Cytogram of FSC and SSC (morphological parameters, expressed in arbitrary units, AU) of *P. multiseries*. B. Cytogram of FL1 and FL3 fluorescence of *P. multiseries*. FL1 is green fluorescence due to SYTOX Green and FL3 is red fluorescence due to chlorophyll (AU). R1 are unstained cells (considered as live cells, in red) and R2 are stained cells (considered as dead cells, in green).

probes, Invitrogen, Eugene, OR, USA) prepared at a working solution of 5 µM. A mix of live/dead cells was prepared to confirm that SYTOX Green stained only dead cells (Veldhuis et al., 2001) and to calibrate the measurement. Cells from a dead culture (killed by heating for 15 min at 100 °C) were mixed with those from a live culture to give a range of 0–100% dead cells (increments of 10%) and stained with SYTOX Green at 0.1 µM (final concentration) for 30 min. The percentage of measured dead cells (those stained with SYTOX Green) was then compared to the theoretical percentage of dead cells present in the mixture.

2.2.3. Metabolic activity

To assess metabolic activity, esterase activity was measured using fluorescein diacetate (FDA, Molecular probes, Invitrogen, Eugene, Oregon, USA). FDA is a probe cleaved by esterases inside the cells, resulting in fluorescein accumulation over time (Jochem, 1999). A 5 mg ml^{-1} stock solution of FDA was prepared by diluting the commercial powder in DMSO. A fresh 300 µM working solution was prepared before each experiment by adding stock solution directly to distilled water cooled on ice. The working solution was kept in darkness and on ice during the experiment and was agitated to prevent the formation of aggregates.

2.2.4. Lipids

To assess intracellular lipid content, two probes were tested on *P. multiseries*. A 10 mM stock solution of BODIPY 493/503 (4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacene, Molecular probes, Invitrogen, Eugene, OR, USA) was made by

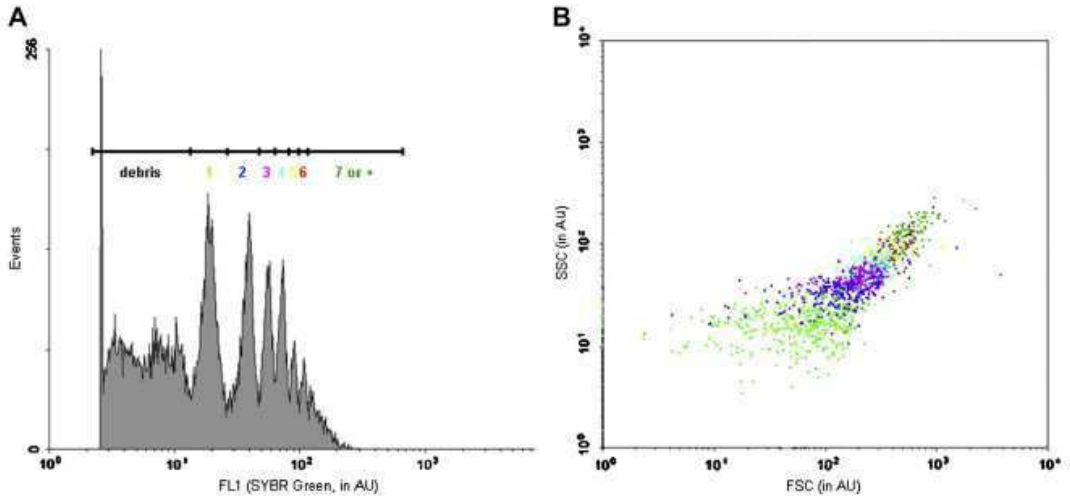

Fig. 2. Bacteria stained with SYBR Green and propidium iodide. A. Histogram of FL1 (green) fluorescence of bacteria; 1 to 7 represent aggregates of 1–7 or more bacteria. FL1 is green fluorescence due to SYBR Green. B. Cytograms of morphological parameters of bacteria (FSC and SSC, expressed in arbitrary units, AU). Each color represents one aggregate size (light green = one bacteria, dark blue = 2 bacteria, pink = 3 bacteria, light blue = 4 bacteria, yellow = 5 bacteria, red = 6 bacteria, dark green = 7 or more bacteria). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

diluting the commercial powder in DMSO. A 1 mM working solution was then prepared by a 10-fold dilution of the stock solution in distilled water. A 1 mg ml^{-1} stock solution of NR (NR, Sigma, St. Louis, MO, USA) was prepared by diluting 100-fold the commercial powder in acetone and then 10-fold in distilled water to obtain a working solution of 0.1 mg ml^{-1}.

Each of these measurements had to be optimized for *P. multiseries*. Thus, final probe concentrations and incubation times were chosen following two rules: (i) the concentration had to be as low as possible to avoid toxic effects of the probe itself (and of the DMSO contained in stock solutions of the probes); and (ii) staining had to be homogeneous (all of the cells had to be stained or only the dead cells for SYTOX Green), relatively stable over time and reproducible between analytical replicates. For each probe, fluorescence measurements were performed every 5 min for 1 h on the FL1 (or FL2 for NR) channel of the flow cytometer. Concentrations of 0.5, 1.0, 2.5 and 5.0 μg ml^{-1} were tested for NR; 1.0, 2.5, 5.0 and 10 μM for BODIPY; 0.75, 1.50 and 3.00 μM for FDA and 0.025, 0.05, 0.1 and 0.2 μM for SYTOX Green.

Quantum yield (QY), a measurement of the efficiency of photosynthesis, was measured using an AquaPen-C AP-C 100 (Photo Systems Instruments, Czech Republic) PAM fluorometer. $QY = (F_m - F_0)/F_m$, where F_0 and F_m are the minimum and maximum fluorescences of cells, respectively, after 30 min of dark adaptation. To ensure that there was no background fluorescence, *P. multiseries* supernatant and f/2 medium were used as blanks.

DA was quantified using the ASP ELISA kit (Biosense Laboratories, Bergen, Norway), according to the manufacturer's protocol. Cultures (cells and supernatant) were sonicated and filtered at 0.22 μm to measure total DA. Supernatant (culture filtered at 0.22 μm) was used to measure dissolved DA. Intracellular DA was measured by subtracting dissolved DA to total DA.

2.3. Monitoring the physiology of P. multiseries over a growth cycle

Six *P. multiseries* cultures of the same strain were sampled every day from day 4 to day 21. The following were assessed on each sampling day: *P. multiseries* morphology, concentration and mortality, bacterial concentration and mortality, total and dissolved DA concentrations, quantum yield, chlorophyll fluorescence, intracellular lipid content and metabolic activity. Growth rate was measured during exponential phase following the formula: $\mu\ (d^{-1}) = \ln(N1/N0)/\Delta t$ (in days). Fluorescence measurements were performed using the optimal concentrations obtained in the previous experiments: 0.1 μM SYTOX Green, 3 μM FDA, 1 μg ml^{-1} NR and 10 μM BODIPY. FDA measurements were performed precisely after 6 min of incubation, and SYTOX Green and BODIPY measurements after 30 min. Bacteria were stained with SYBR Green and PI for 15 min.

2.4. Statistics

Results were analyzed statistically with simple regressions, one-way ANOVA with time as the main factor and principal component analysis (PCA) followed by a factorial plan. For all statistical results, a probability of $p < 0.05$ was considered significant. Statistical analyses were performed using StatGraphics Plus (Manugistics, Inc, Rockville, MD, USA).

3. Results

3.1. Optimization of probe concentrations

3.1.1. Mortality

A 0.1 μM final concentration of SYTOX Green allowed a good distinction between dead and live cells (Fig. 1). Incubation time was optimal at 30 min. The best correlation ($y = 0.95x$, $R^2 = 0.99$, $P < 0.01$) between the measured and theoretical percentages of dead cells in the mixtures of dead/live *P. multiseries* was established at a SYTOX Green concentration of 0.1 μM, which was therefore applied for further analyses.

3.1.2. Bacteria

Free-living bacteria are able to form aggregates that can be distinguished after SYBR Green staining (Fig. 2A). Each aggregates exhibited fluorescence that was equivalent to the fluorescence of one bacteria × number of bacteria in the aggregate. The number of total free-living bacteria can thus be deduced and measurements of FSC and SSC can be done for each aggregate size (Fig. 2B).

3.1.3. Metabolic activity

Concentrations of FDA lower than 3.0 μM exhibited low fluorescence, indicating that there was little accumulation of the probe (data not shown). At 3.0 μM, fluorescein accumulated within the cells (Fig. 3I), in a linear manner during the first 15 min ($y = 35.9x + 133.1$, $R^2 = 0.9964$, $p < 0.001$), and then reached a plateau (Fig. 4). For further analyses, fluorescein accumulation was measured after 6 min of staining within the linear part of the curve.

3.1.4. Lipids

A final concentration of 10 μM BODIPY 493/503 and 1 μg ml^{-1} of NR enabled the best staining of all cells (one distinct population of cells, not a diffuse cloud of cells, could be seen on the cytograms, data not shown). After 30 min, all cells were well stained and fluorescence was stable (Fig. 3B, C, E, F).

3.2. Monitoring of morphofunctional characteristics during P. multiseries growth

The exponential growth phase of *P. multiseries* started after a 7-day lag phase, giving a growth rate of $0.24 \pm 0.01\ d^{-1}$, and then the stationary phase was reached after day 17 (Fig. 5). A maximal concentration of $\sim 8 \times 10^4$ cells ml^{-1} was observed at days 17 and 19, after which the cell concentration rapidly declined.

Bacteria within the *P. multiseries* culture started their exponential growth phase on day 7 and were still growing steadily

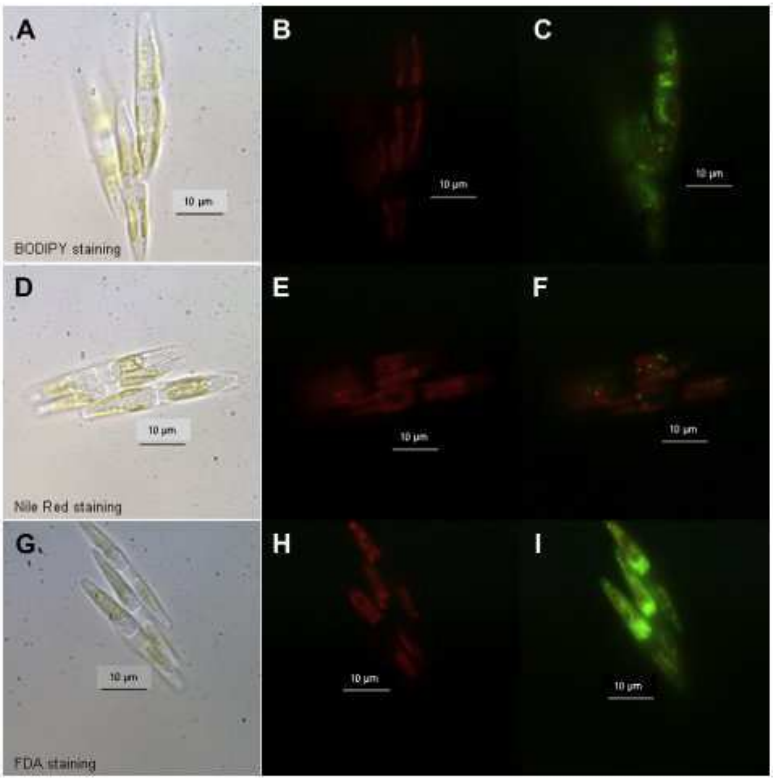

Fig. 3. Photomicrographs of *P. multiseries* cells in white light (A, D, G), epifluorescence light with filter "BP 515/560/BS 580/LP 590" (B, E, H) and filter "BP 450-490/BS 510/LP 515" (C, F, I). A, B, C) Cells stained with BODIPY. D, E, F) Cells stained with NR. G, H, I) Cells stained with FDA. Scale bar = 10 µm.

until the end of the experiment (Table 1), exhibiting a growth rate of 0.07 ± 0.01 d^{-1}. The bacteria/*P. multiseries* cell ratio decreased during the exponential phase of *P. multiseries*, remained stable between days 14–20 and then increased again on the last day of the experiment, when *P. multiseries* numbers declined (Fig. 5, Table 1). Proportions of bacteria in aggregates of one, two or more cells did not change with growth phases. The percentage of dead bacteria decreased between days 4 and 12 (from 5.8% ± 0.6 to 2.0% ± 0.2) and then remained stable between 1.9 and 2.5% until day 21 (Table 1). Values of FSC and SSC for the bacterial community (free-living bacteria that were not forming aggregates) decreased steadily during the course of *P. multiseries* culture (Table 1).

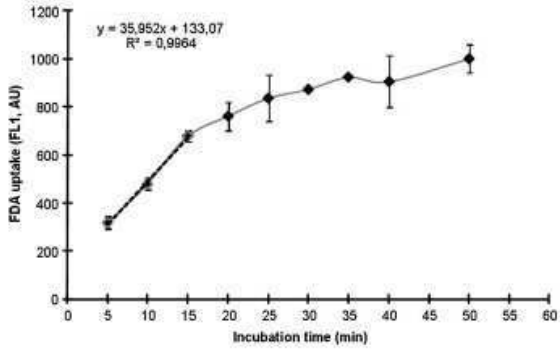

Fig. 4. Green fluorescence of *P. multiseries* cells (in arbitrary units, AU) stained with 3.0 µM of FDA and measured on FL1 detector of a flow cytometer ($n = 3$, mean ± SD).

The percentage of dead *P. multiseries* cells averaged 28% throughout the entire experiment (Table 1) and decreased from 30.3% on day 12 to 18.9% at day 16, after which it increased to 43.8% on day 20 (stationary phase). FSC values for *P. multiseries* continuously decreased during the experiment, almost linearly with culture age ($R^2 = 0.76$, $p < 0.01$). SSC values decreased until day 13 (mid-exponential phase) and became stable between day 13 and the end of the experiment (Table 1).

Total DA in the *P. multiseries* culture, expressed as pg ml^{-1}, increased steadily from day 7 (200 ± 21 pg ml^{-1}) until day 14 (798 ± 164 pg ml^{-1}) during exponential growth. Total DA in the culture then decreased sharply, reaching a concentration below 100 pg ml^{-1} on day 21. Total DA content was highest on days 13 and 14 during the mid-exponential phase, and decreased steadily after day 14, when it reached late-exponential phase and stationary phase (Fig. 5). The amount of dissolved DA was low and remained constant throughout the culture, from 41.3 (±2.9) pg ml^{-1} on day 6 to 103.0 (±7.3) pg ml^{-1} on day 12, representing 11–40% of total DA.

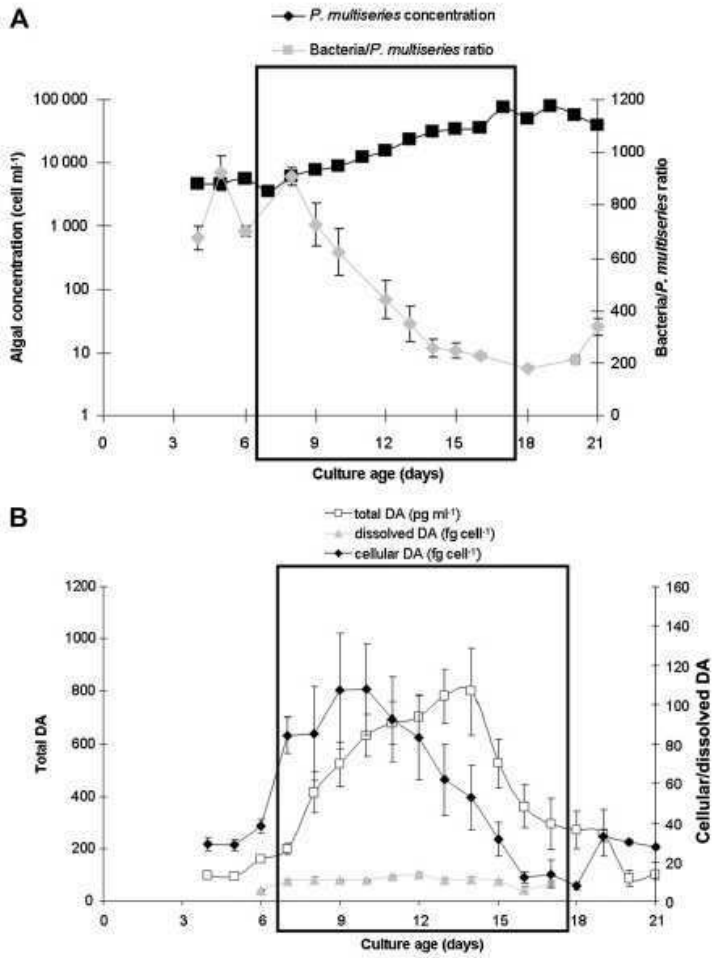

Fig. 5. A. *P. multiseries* growth curve (*y*-axis) and bacteria/*P. multiseries* ratio (*z*-axis, $n = 6$, mean ± SE). The exponential growth phase of *P. multiseries* is framed with a black-lined rectangle. B. Concentration of total for the z-axis DA in the whole culture (*y*-axis, pg ml^{-1}) and cellular DA in fg $cell^{-1}$ (z-axis, $n = 6$, mean ± SE). Exponential growth phase of *P. multiseries* is framed with a black-lined rectangle.

FL3 values (related to chlorophyll content) were measured on live cells, discriminated from dead cells using SYTOX Green staining. FL3 values sharply decreased from day 4–6, remained stable between days 6 (590 ± 12) and 9 (593 ± 10) and then slightly decreased from day 9 to day 20 (506 ± 12), stationary phase, Fig. 6). Quantum yield (QY) values increased between days 5 (0.46 ± 0.01) and 8 (0.59 ± 0.01), became relatively stable until day 14 (0.62 ± 0.00), and then decreased in mid-exponential phase after day 14 (Fig. 6). Day 11 exhibited a significant decrease in both FL3 and QY. Supernatant and media did not exhibit QY values or were below the detection threshold of the fluorometer.

The metabolic activity of the *P. multiseries* cells, as measured with the FDA assay after 6 min of incubation, increased rapidly from day 6 to day 7 and then just as rapidly decreased after day 7, to values just below the initial level, on day 9 (Fig. 7). The percentage of stained cells after 6 min of incubation increased between day 8 (72.5% ± 1.6) and 16 (87.9% ± 1.0) and decreased on day 20 (74.7% ± 5.4). The percentages of live cells, as measured with the SYTOX Green and FDA assays, were significantly correlated, even though the correlation remained quite weak ($R^2 = 0.67$ $p < 0.001$, Fig. 7).

The amount of intracellular lipids, interpreted from BODIPY fluorescence, increased between days 4 and 6, decreased from days 6 to day 14 (during the exponential phase), stayed stable until day 16 and finally increased during the stationary phase (Fig. 8). NR fluorescence, the traditional indicator of lipid content, decreased between days 9 and 20, with one higher fluorescence value on day 11 (Fig. 8).

PCA showed that DA content of cells (total DA) had coordinates very close to those of NR, SSC and the bacteria/*Pseudo-nitzschia* ratio, knowing that components 1 and 2 explained 74% of the variability (Fig. 9). FSC and BOPIDY uptake were also closely correlated with these previous

Table 1
P. multiseries and associated bacterial concentration (of live cells), morphological parameters (FSC and SSC, in arbitrary units), percentage of dead *P. multiseries* measured using SYTOX Green and percentage of dead bacteria assessed using SYBR Green, propidium iodide double staining ($n = 6$, mean ± SE).

Day	*P. multiseries*								Bacteria							
	FSC (AU)		SSC (AU)		% dead cells		Concentration (cell ml^{-1})		FSC (AU)		SSC (AU)		% dead cells		Concentration (10^6 bact ml^{-1})	
	Mean	±SE	Mean	±SE	Mean	±SE	Mean	±SE	Mean	±SE	Mean	±SE	Mean	±SE	Mean	±SE
4	196.3	2.6	76.8	0.7	25.0	1.2	4526	190	226.4	10.8	23.5	0.7	5.8	0.6	3.01	0.07
5	203.5	0.7	76.9	1.4	27.9	1.7	4574	255	207.3	9.1	23.1	0.5	4.2	0.3	4.14	0.15
6	186.0	1.3	80.0	1.8	22.6	0.9	5452	214	179.1	5.2	22.9	0.4	4.0	0.3	3.80	0.13
7	197.6	2.7	71.2	1.6	32.7	0.7	3533	93								
8	183.7	2.9	72.6	1.5	30.6	0.8	6226	275	177.4	2.8	23.0	0.2	3.5	0.3	5.61	0.04
9	185.2	1.5	72.1	1.0	32.6	2.0	7711	677	179.1	4.6	24.5	0.3	2.6	0.3	5.33	0.23
10	190.5	2.0	67.5	1.3	28.5	2.7	9059	1179	168.9	7.5	24.3	0.4	2.7	0.2	5.15	0.20
11	190.5	1.6	62.9	0.7	54.5	9.4	12,393	1730								
12	186.3	1.4	63.3	0.9	30.3	3.8	15,863	2716	127.6	5.0	23.2	0.4	2.0	0.2	6.09	0.21
13	180.4	1.3	59.1	0.8	22.8	3.0	23,837	5343	186.5	18.7	23.0	1.5	1.9	0.1	6.60	0.12
14	177.4	2.0	58.7	1.1	22.9	2.9	30,726	6223	171.4	26.7	20.2	0.6	2.2	0.2	6.80	0.21
15	173.5	0.9	59.7	1.3	19.1	0.7	33,796	4967	193.3	7.7	21.1	0.7	2.2	0.1	7.71	0.23
16	171.1	1.9	60.0	1.3	18.9	1.6	36,148	2064	225.9	18.1	22.7	1.4	2.2	0.1	8.19	0.16
17	185.4	0.9	57.3	1.2			77,322	3718								
18	173.9	3.1	60.2	1.3	27.4	4.2	49,222	4818	99.2	5.9	19.3	0.7	1.9	0.1	8.72	0.55
19	174.9	2.1	56.3	0.7			78,730	10,135								
20	168.8	1.3	60.3	0.3	43.8	5.5	55,889	8682	177.9	8.6	19.0	0.7	2.0	0.1	11.31	0.97
21	163.7	2.3	59.7	0.5			40,163	2226	25.1	0.7	17.3	0.4	2.1	0.2	13.30	0.81

parameters, indicating that increased DA production was associated with a higher intracellular lipid content. A factorial plan (Fig. 10) was developed from the previous PCA, plotting the age of the *P. multiseries* culture in exponential and stationary phases from day 9 to day 20. Days followed a consistent trend, from high component 1 and low component 2 (i.e. high lipid concentration, high DA content, high cell/bacteria ratio, low esterases activity, etc.), towards lower component 1 and higher component 2 (i.e. high esterases activity, low DA content and low lipid concentration, etc.). Day 20 was the only day which did not follow this trend at the extremities of the factorial plan (extremely low components 1 and 2, i.e. driven mainly by high *P. multiseries* mortality and a high number of bacteria).

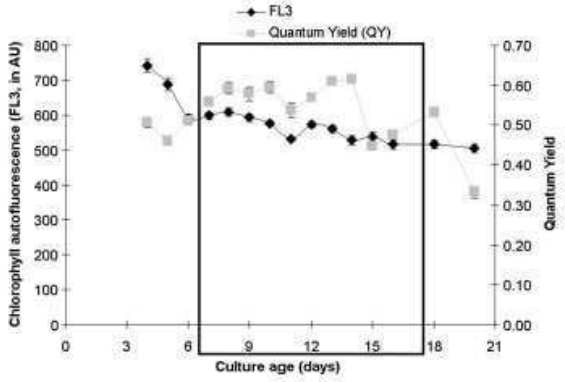

Fig. 6. Chlorophyll fluorescence (FL3, in arbitrary units, AU, *y*-axis) and Quantum Yield (QY, *z*-axis) of live *P. multiseries* cells as a function of culture age. FL3 was measured using flow cytometry on live cells, as determined by SYTOX Green staining ($n = 6$, mean ± SE). The exponential growth phase of *P. multiseries* is framed with a black-lined rectangle.

4. Discussion

The primary aim of this study was to test and optimize several methods and probes for assessing *Pseudo-nitzschia* physiological status. The percent of cell mortality in the cultures was determined using SYTOX Green, which only penetrates cells that have lost their membrane integrity and are thus considered dead cells (Veldhuis et al., 1997). A final concentration of 0.1 µM was optimal for staining *P. multiseries* dead cells and is in good agreement with those found in the literature for other phytoplankton species (Veldhuis et al., 2001; Binet and Stauber, 2006; Ribalet et al., 2007; Miller-Morey and Van Dolah, 2004; Lawrence et al., 2006).

Fluorescein diacetate (FDA) has previously been used to measure metabolic activity (Jochem, 1999; Regel et al., 2002; Brookes et al., 2000) as well as viability of microalgae (Lawrence et al., 2006; Dorsey et al., 1989; Jansen and Bathmann, 2007). It penetrates the cells passively and, once within a cell, is hydrolyzed by non-specific esterases into fluorescein and two acetate molecules. The more metabolically active the cells, the more esterases they produce, resulting in a greater amount of fluorescein accumulation within the cells. The probe will not be cleaved within dead cells, as esterases are inactive. Moreover, if the probe is hydrolyzed by any remaining esterases, fluorescein will leak out of the cells, as the membranes are permeable. Thus, unstained cells are considered to be dead cells. In the literature, measurement of fluorescein released from FDA inside the cells most often occurs between 5 and 20 min of incubation (Jochem, 1999; Regel et al., 2002; Dorsey et al., 1989; Jamers et al., 2009). FDA only accumulated linearly during the first 15–20 min, as previously observed by Gilbert et al. (1992). Accordingly, based on our results and supported by the above publications, measurements were performed after 6 min of

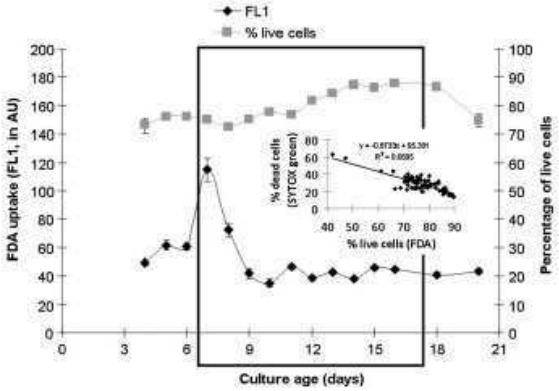

Fig. 7. FDA uptake (FL1 fluorescence of live cells, *y*-axis) and percentage of *P. multiseries* live cells stained by FDA (*z*-axis) and detected using flow cytometer FL1 detector ($n = 6$, mean ± SE). Exponential growth phase of *P. multiseries* is framed with a black-lined rectangle. Correlation between the percentages of live cells measured with the SYTOX Green and FDA assays is indicated in the small graph (in arbitrary units, AU).

incubation. A final concentration of 3 μM was optimal for this assay and is consistent with some publications (Dorsey et al., 1989; Gilbert et al., 1992), but lower than others (Regel et al., 2002; Jamers et al., 2009). Higher concentrations of FDA were not tested, as 3 μM provided satisfactory staining, and higher concentrations of FDA and DMSO may become toxic to the cells.

BODIPY 493/503 and NR were tested to localize and quantify intracellular lipids in *P. multiseries* cells. NR has been used traditionally to stain lipids of microalgae (Cooksey et al., 1987), whereas this is the first time that BODIPY 493/503 has been used to study microalgal lipids. NR fluorescence of microalgal lipids, measured by FCM, has been shown to be linearly correlated with the lipid content of cells (de la Jara et al., 2003). Lipids of *P. multiseries*, revealed by BODIPY

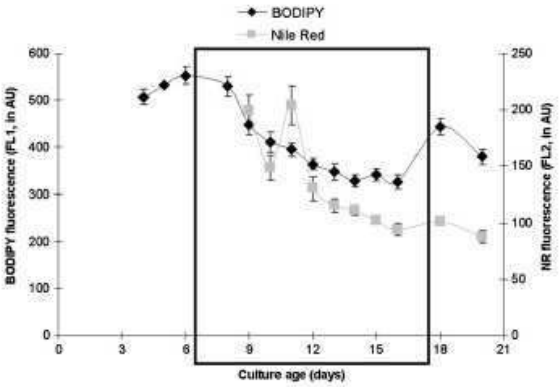

Fig. 8. Green and orange fluorescences of *P. multiseries* cells stained with BODIPY 493/503 and NR (indicators of lipid content) and detected by the FL1 (*y*-axis) and FL2 (*z*-axis) detectors, respectively, on a flow cytometer, in arbitrary units ($n = 6$, mean ± SE). The exponential growth phase of *P. multiseries* is framed with a black-lined rectangle.

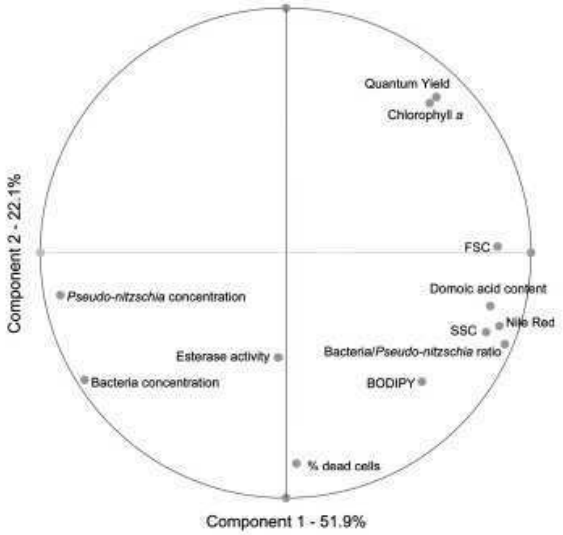

Fig. 9. Principal component analysis (PCA) plot of all physiological measurements between days 9 and 20 (D9 to D20) of the *P. multiseries* culture ($n = 52$).

and NR, were observed to form vacuoles inside the cells (Fig. 3), and NR gave a lower fluorescence intensity than BODIPY. These vacuoles are likely to contain reserve lipids, as BODIPY and NR are reported to stain neutral lipids (Gocze and Freeman, 1994). Such vacuoles have previously been described within microalgae (Eltgroth et al., 2005; Liu and Lin, 2001; Remias et al., 2009; Cooper et al., 2010), although lipid-staining BODIPY and NR did not reveal a specific distribution of these vesicles. Both BODIPY and NR were used to quantify intracellular lipid contents by FCM, in the green (FL1) and orange (FL2) channels respectively. In the present study, NR was used at a final concentration of 1 μg ml^{-1}, which is the same as that used in previous studies on microalgae (Chen et al., 2009, 2010; Liu et al., 2008; Huang et al., 2009; McGinnis et al., 1997). BODIPY was used at a final concentration of 10 μM. This concentration allowed the detection of subtle variations in the intracellular lipid content of *P. multiseries* grown, for example, in culture media with or without nitrate (data not shown), whereas lower concentrations did not. Higher concentrations were not tested, as 10 μM provided satisfying staining, and higher concentrations of BODIPY and DMSO may become toxic to the cells. The concentration used was 100 times higher than that used for fungus (Saito et al., 2004), but in agreement with those on human muscle (Wolins et al., 2001) and lower than that used on amoeba (Kosta et al., 2004).

The development of these methods allowed the physiological status of *P. multiseries* cells to be monitored over a complete growth cycle. The lag phase of *P. multiseries* lasted 7 days, which is long compared to other studies on the same species but not on the same strain (Thessen et al., 2009; Lundholm et al., 2004; Kudela et al., 2003; Kotaki et al., 1999; Bates et al., 2000). The *P. multiseries* growth rate

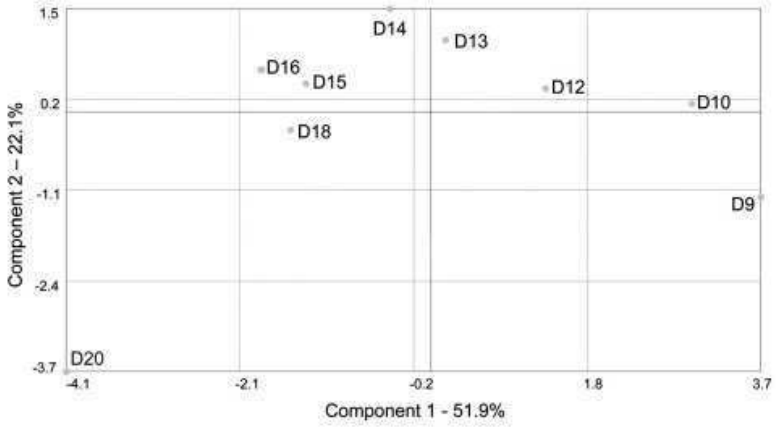

Fig. 10. Factorial plan issued from the previous PCA and plotting days of culture of *P. multiseries*, from day 9 to day 20 (D9 to D20, $n = 52$).

(0.24 ± 0.01 d^{-1}) was lower than those previously reported in the literature (Thessen et al., 2009; Lundholm et al., 2004; Kudela et al., 2003; Kotaki et al., 1999; Bates et al., 2000). This might be explained by the age of the isolate (isolated in 2007, more than 2 years ago) and the short cell length (~20 μm); Amato et al. (2005) reported a slight decrease in the growth rate of *P. delicatissima* with a decrease in apical cell length. Culture conditions were the same or close to those in studies using *Pseudo-nitzschia* cultures (media, irradiance and temperature) and thus could not explain differences in growth rates.

FSC and SSC values of *P. multiseries* decreased during the entire experiment, by 17% and 22%, respectively. FSC and SSC resulted from the diffraction of the laser by the cell surface. Their decrease in *P. multiseries* may be related to changes in external morphology, cell size and internal cell complexity. During growth, cells undergo asexual reproduction and thus decrease in cell length. FSC and SSC values were, however, similar to values measured over the last year (data not shown) at both the beginning (during the lag phase) and end of experiments. This indicates that FSC and SSC values changed very little over the last year, possibly because this strain isolated in 2007 was already quite old. Inoculation of *P. multiseries* into a new medium resulted in a return to high FSC and SSC values. Because diatoms cannot increase their cell size, the changes in FSC and SSC values are more likely related to both surface membrane and cytoplasmic modifications than cell size modifications, thus modifying the diffraction of the laser. This hypothesis is based on the correlation between SSC and both BODIPY and NR fluorescences ($R^2 = 0.77$ and 0.64, respectively, at $p < 0.01$). It is possible that when cells had a lot of lipid vesicles within their cytoplasm, this increased cell complexity was reflected by changes in FSC and SSC values.

Bacterial community counts and morphological changes within *Pseudo-nitzschia* cultures were estimated for the first time by FCM. In this microalgal culture, the growth rate of the bacteria was 0.07 d^{-1}, which remained constant over the course of the experiment; the bacteria did not reach stationary phase during the 20 days of the experiment. This growth rate is in the lower range of bacteria grown in adapted culture media, that can grow from 0.01 h^{-1} (Kemp et al., 1993) to 1.5 h^{-1} (Makino et al., 2003). These differences may be due to competition with *P. multiseries* for some nutrients, or the fact that they may not have all the nutrients they need and that are usually added in agar plates. The highest bacteria/*P. multiseries* ratios were measured during the lag phase (days 4–7) and at the beginning of the exponential phase (days 7 and 8). Bacteria measured are the free-living bacteria contained in the medium; however, some bacteria can also be attached directly to *P. multiseries* cells (Kaczmarska et al., 2005), and these attached bacteria were not taken into account (their signal was confounded within these of *P. multiseries*). The decrease in the number of bacteria per *P. multiseries* cell during the exponential phase of *P. multiseries* (from 922 to 180) is explained by faster growth rate of *P. multiseries* compared to bacteria. The increase in the bacteria/*P. multiseries* ratio during the senescent phase of *P. multiseries* may be a result of bacteria taking advantage of organic materials released from dead *P. multiseries* cells (Kaczmarska et al., 2005). Stewart et al. (1997) found between 7 and 10 bacteria per *P. multiseries* cell, which is about 20–80 times lower than our values. This difference may be explained by (i) a high residual percentage of dead *P. multiseries* cells present during the entire experiment, or (ii) the age of our isolate, which provided sufficient time (two years) for the bacterial community to adapt to culture conditions of *P. multiseries*. Differences found in bacterial communities over time in culture for non-toxic *Pseudo-nitzschia pungens* support this possibility (Sapp et al., 2007), but Wrabel and Rocap (2007) found no shifts in bacterial assemblages in a *Pseudo-nitzschia* culture over its initial nine months. Nevertheless, the shift in the bacterial community may appear after 9 months in culture. FSC and SSC values of the bacterial community decreased during the experiment. These values are related to size and complexity of bacterial cells. This may reflect a shift in species composition

of the bacterial community to smaller bacteria or a decrease in bacterial cell size. Between 1.9 and 5.8% of the bacteria in our cultures were dead, with the highest percentage at day 4. The percentage of dead bacteria remained quite low (1.9–2.7%) until the end of the experiment, as they were still in exponential phase.

Values of FL3 (related to the chlorophyll content) decreased slightly during the entire experiment, with the greatest decrease between days 4 and 6. The chlorophyll content of *P. multiseries* decreased only slightly during the exponential phase. Nevertheless, cells with more chlorophyll may not necessarily have the most efficient photosynthesis. Indeed, QY, a measure of the efficiency of photosynthesis, was not well correlated with FL3 values, as QY decreased during the stationary phase when FL3 remained high. QY increased at the beginning of the exponential phase and remained high during the remaining exponential phase, with cells having efficient photosynthesis with a lot of energy produced. Such an increase in QY during the exponential phase has been shown for other microalgal species, e.g. *Symbiodinium* sp. (Rodriguez-Roman and Iglesias-Prieto, 2005), and is currently used as a measure of algal culture health. As the QY value is not affected by the percentage of dead cells in the cultures (Franklin et al., 2009), it can be speculated that at the end of the stationary phase, live *P. multiseries* cells still contained high amounts of chlorophyll, but with poor photosynthetic efficiency.

During the entire experiment, the percentage of dead *P. multiseries* cells was relatively high, ranging from 19% to 54%. Nevertheless, our cultures reached a maximum cell concentration of 8×10^4 cells ml^{-1}, which is consistent with previous studies (Mengelt and Prézelin, 2002; Bates and Richard, 1996; Lewis et al., 1993; Kotaki et al., 1999), but lower than results of most studies (Bates and Richard, 1996; Kotaki et al., 1999; Mengelt and Prézelin, 2002), suggesting that our cultures were not in good health, which also explains the low growth rate and high percentage of dead cells. Generally, in healthy and young cultures of *Pseudo-nitzschia* sp., the percentage of dead cells has been described under 5% (Mengelt and Prézelin, 2002). The increase in dead cells at the end of the experiment may be due to limitations in nutrients and associated with the beginning of the stationary phase. Such a consistently high percentage of dead cells in the culture may be explained by the age of the isolate. The percentage of dead cells assessed with FDA was significantly, but not perfectly, correlated ($R^2 = 0.67$, $p < 0.01$) with those obtained with SYTOX Green, and appeared slightly lower than when measured with SYTOX Green. Cells can have a compromised cell membrane and be considered as dead when assessed with SYTOX Green, but they may still have active esterases. These false-positive cells (dead but stained with FDA) have been shown to represent 1.6% of total cells of *Chlamydomonas reinhardtii* (Jamers et al., 2009). Such differences between SYTOX Green and FDA have also been previously observed in *Heterosigma akashiwo* (Lawrence et al., 2006). Using these two probes not only provides the percentage of dead versus live cells, but also provides an indication of the way cells are dying. In our cultures, cells most likely died by loss of membrane integrity prior to inactivation of esterases, which was also observed by Lawrence et al. (2006) in cultures of *H. akashiwo*. Thus, SYTOX Green and FDA provide useful information, and both could be used in physiological measurements.

Lipid-related fluorescence assessed with BODIPY was high during the lag phase, indicating that the cells contained energy stored as neutral lipids. BODIPY fluorescence decreased throughout the entire exponential phase, suggesting that cells were using these stored lipids to grow in addition to the energy produced by photosynthesis. Cells stopped growing at the stationary phase, and energy was once again stored as lipids, as evidenced by the increase in BODIPY fluorescence. Although no data are available between days 4 and 6, NR fluorescence decreased during the remainder of the experiment, with the exception of a high value on day 11. There was a weak correlation between BODIPY and NR fluorescence during the exponential phase (between days 7 and 18; $R^2 = 0.65$, $p < 0.01$). During the stationary phase, however, BODIPY fluorescence is higher than that of NR, which confirms that these two probes may not actually stain the same compounds during that period. This emphasizes the importance of using both lipid probes. These differences may be explained by the chemical properties of the two probes. BODIPY 493/503 stains intracellular lipids more effectively than NR, with a higher sensitivity and lower background (Kacmar et al., 2006). BODIPY 493/503 also stains intracellular lipid droplets more specifically than does NR (Gocze and Freeman, 1994). NR is an uncharged hydrophobic molecule whose fluorescence is strongly influenced by the polarity of its environment. Like lipids, NR interacts with many, but not all, native proteins (Sackett and Wolff, 1987) and can undergo changes in fluorescence intensity when it binds to certain proteins (Brown et al., 1995). The fact that NR binds proteins may explain its lower sensitivity to small variations in lipid content, as measured by BODIPY. This is especially evident during the stationary phase, when differences in lipid staining were observed between the two probes. Thus, the combined use of BODIPY and NR probes is of interest, as they may reflect different physiological changes.

The maximum of total DA per cell was observed on days 9 and 10, in early exponential phase, and it decreased during the remainder of the exponential phase and the stationary phase. The same pattern of DA production has been observed for *Pseudo-nitzschia calliantha* (Besiktepe et al., 2008) and *Pseudo-nitzschia pseudodelicatissima* (Pan et al., 2001), where maximum DA production was observed during the early exponential phase. All studies of *P. multiseries*, however, have found maximum DA production during the stationary phase (Kotaki et al., 1999; Bates et al., 2000; Lewis et al., 1993; Osada and Stewart, 1997). It is possible that old cultures of *P. multiseries* exhibit a shift in DA production from stationary phase to early exponential phase, which is difficult to prove, as no strain has ever been studied throughout its lifetime in the laboratory. Moreover, strains exhibiting DA production during early exponential phase seem to have a lower DA content per

cell (Besiktepe et al., 2008; Pan et al., 2001). In our study, total cellular DA varied between 0 and 192 fg cell^{-1}, which is low compared to previous studies on *P. multiseries*, where DA attained 1.2–45 pg cell^{-1} (Bates et al., 2000; Thessen et al., 2009). Our values are more consistent with those of *P. calliantha* (Álvarez et al., 2009) and *P. pseudodelicatissima* (Pan et al., 2001), which had a maximum toxicity of 10 and 36 fg cell^{-1}, respectively, but these species have a smaller cell volume. Our strain of *P. multiseries* was very short (around 20 µm length here), whereas cells can be 100 µm long just after sexual reproduction, which may explain the low values of DA it produced. DA intracellular content started to decrease from day 11 to the end of the stationary phase. This decrease in DA may also coincide with physiological stress. Unfortunately, bacteria were not measured that day. Nevertheless, day 11 exhibited surprising values for NR, FL3 and QY (i.e. out of the trend). Cells might have undergone stress, with loss of chlorophyll and thus decreased QY; hence, energy was stored under lipid form and DA production was stopped. Dissolved DA was particularly low, but remained constant over time, with cells excreting 11–40% of their total DA. This low DA release may be due to the age of the strain, isolated in 2007, and its consequent smaller size.

DA is a secondary metabolite and is thus believed to be produced when cells have excess energy that is not used for primary metabolism (Bates, 1998). Meanwhile, extra energy is stored as lipids when cells are not able to use it for primary metabolism. In this study, the measure of FDA provided information regarding primary metabolism, and QY (photosynthetic efficiency) was measured to estimate the production of energy. There was no clear relationship between DA production (total or dissolved) and QY or FDA hydrolysis. Conversely, a positive correlation was observed between total DA content and NR after PCA analysis (Fig. 9). Cells of *P. multiseries* seemed to produce more DA when they had more lipids and thus more available stored energy, which is in agreement with some studies (Whyte et al., 1995), but not with others (Pan et al., 1996). Indeed, Pan et al. (1996) hypothesized that DA and lipid synthesis shared some precursors like acetyl-CoA, so that when DA is produced, lipids cannot be stored. Bacteria are also known to play a role in DA production by enhancing DA production through unknown mechanisms (Bates et al., 1995; Stewart et al., 1997). In this study, the ratio of bacteria per *P. multiseries* cell was also weakly correlated with DA content, as the variable coordinates are quite close in PCA analysis ($R^2 = 0.49$, $p < 0.001$, Fig. 9). Cells seemed to produce more DA when more bacteria per *P. multiseries* cell were present in the culture, possibly indicating that more DA was produced either when competition with bacteria was greater or if bacteria produced toxin-enhancing compounds. FSC and BOPIDY uptake was also closely correlated with DA content, SSC, NR and the ratio bacteria/*Pseudo-nitzschia*, indicating that increased DA content is associated with a higher intracellular lipid content. This lipid increase can cause an increase in the amount and/or size of lipid vacuoles within the cells, which could also explain the increase observed in FSC and SSC of the *P. multiseries* cells. A factorial plan (Fig. 10) was developed from the previous PCA, which plots the incubation time of the *P. multiseries* culture, from day 9 to day 20 (values included in the previous PCA). The position of the days included on this factorial plan clearly demonstrates and summarizes our findings: gradual and continuous shift in culture from low algal concentration, high bacteria/algal ratio, large SSC, high lipid and DA content in early stationary phase, towards increasing concentrations, reaching a maximum at the end of the exponential phase, and finally showing a high percentage of dead algal cells and bacteria in late stationary phase.

FCM has been previously used on microalgae, mainly for cell counting or measurement of only one physiological parameter per experiment. Here we developed a set of physiological measurements which provides a more complete description of the physiological status of the microalgae. This technique was applied to one species of *Pseudo-nitzschia* but can be broadened to other microalgal species, whether or not they are toxic or diatoms. Developing cell-based physiological measurements with FCM will help to further our understanding of phytoplankton physiology and its responses to environmental changes, both biotic and abiotic.

Acknowledgements

The authors would like to thank S.S. Bates for his constructive comments and English corrections. This work was supported by the national program EC2CO MicrobiEN, the "Région Bretagne" and the French Ministry of Research (MENRT grant).

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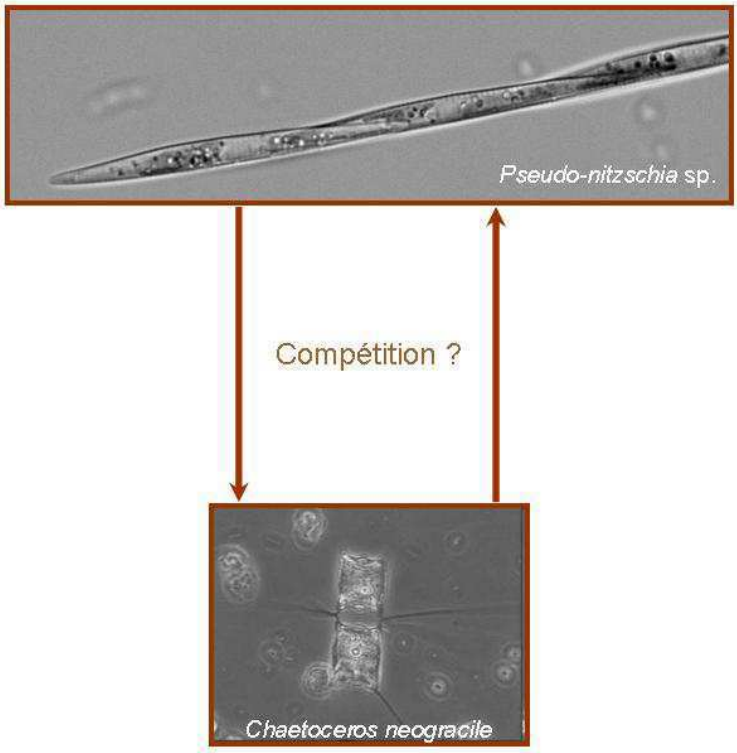
2.4. Conclusion

L'utilisation de sondes fluorescentes a permis de suivre la physiologie de *P. multiseries* tout au long de sa croissance, et surtout d'observer les variations de physiologie au cours des différentes phases de croissance et d'étudier les états physiologiques associés à chaque phase de croissance. Ainsi, en phase exponentielle, les cellules se divisent et utilisent toute l'énergie disponible pour leur métabolisme primaire, très fort en début de phase exponentielle. Les cellules ne stockent donc pas de lipides, et utilisent même leurs lipides de réserve. Elles ne sont pas non plus capables de produire de l'acide domoïque, l'énergie n'étant pas disponible pour le métabolisme secondaire. Leur photosynthèse est par contre très efficace, pour permettre ensuite aux cellules l'acquisition de carbone, nécessaire à la division cellulaire. En phase stationnaire, à l'inverse, la photosynthèse devient moins efficace, les cellules ne se divisent plus et stockent donc l'énergie accumulée sous forme de lipides de réserve. La synthèse d'acide domoïque est aussi forte en fin de phase exponentielle-début de phase stationnaire, quand l'énergie disponible peut enfin être allouée au métabolisme secondaire. Cette production démarre après un pic de forte activité des estérases. Arrivées en phase stationnaire, les cellules, généralement limitées en CO_2 et en silice, commencent à mourir.

Les mesures physiologiques mises au point dans ce chapitre permettent donc de définir l'état physiologique des cellules, en étudiant non seulement leur photosynthèse, mais aussi leur métabolisme primaire, l'état de leurs réserves énergétiques et leur production d'acide domoïque. Ces mesures ne sont pas uniquement applicables à *Pseudo-nitzschia* mais potentiellement à toutes les espèces de microalgues assez petites pour passer au cytomètre en flux (< 80 µm). Elles ont ainsi été appliquées à d'autres espèces dans le cadre d'une collaboration avec Dr. Dianne Jolley de l'Université de Wollongong, Australie. Le métabolisme des cellules de *Pseudo-nitzschia* variant probablement en fonction des conditions de cultures, les techniques pour mesurer l'état physiologique des cellules mises au point dans ce chapitre sont appliquées à des cellules subissant des stress biotiques et abiotiques dans les études présentées dans les chapitres suivants.

COMPETITION ENTRE *PSEUDO-NITZSCHIA MULTISERIES* ET UNE AUTRE DIATOMEE

3



Article :

Lelong A., Hégaret H., Soudant P. How does *Pseudo-nitzschia multiseries*, a toxic diatom, compete with diatoms of the *Chaetoceros* genus? En préparation pour soumission à Applied and Environmental Microbiology.

3.1. Préambule

Des milliers, voire des millions, d'espèces de microalgues peuplent l'Océan mondial. Même si l'Océan mondial couvre une vaste surface, les différentes espèces ne sont jamais seules et plusieurs cohabitent dans une même niche écologique. Si les espèces n'ont pas toutes les mêmes besoins, ce qui leur permet de cohabiter, elles peuvent à un moment donné entrer en compétition, soit pour l'acquisition des nutriments (par exemple Yoshiyama et al., 2009), soit pour l'espace (par exemple Smayda, 2002) ou encore pour la lumière (par exemple Yoshiyama et al., 2009). La diatomée *Pseudo-nitzschia* fait partie de ces microalgues capables de former des efflorescences, pas forcément mono spécifiques (Quijano-Scheggia et al., 2008). Elle est donc parfois capable de l'emporter en terme de concentration et de biomasse sur les autres espèces phytoplanctoniques, qui par ailleurs survivent à ces efflorescences de *Pseudo-nitzschia* par exemple (REPHY, Ifremer). Pour ce faire elle a dû développer des mécanismes spécifiques. Les cellules de *Pseudo-nitzschia* n'étant pas allélopathiques (Lundholm et al., 2005), l'adaptation est très probablement physiologique et la production d'acide domoïque pourrait jouer un rôle pouvant favoriser *Pseudo-nitzschia* au détriment d'autres espèces. L'acide domoïque est en effet un chélatant du fer et du cuivre (Rue and Bruland, 2001) et pourrait donc être produit pour acquérir fer et cuivre aux dépens de ses compétiteurs, microalgues comme bactéries. L'acide domoïque étant produit en conditions non limitantes, son rôle n'est probablement pas limité à l'acquisition de métaux en conditions limitantes ou toxiques.

Nous avons donc voulu savoir comment *Pseudo-nitzschia multiseries* pouvait s'adapter à la présence de *Chaetoceros neogracile*, une diatomée dont on retrouve le genre sur les côtes bretonnes et qui forme des efflorescences à la même période que *Pseudo-nitzschia* spp. (REPHY). Cette adaptation passe-t-elle par la production d'acide domoïque et/ou par des modifications physiologiques ? La physiologie et la production d'acide domoïque de *P. multiseries* ont donc été suivies en mono- et co-cultures avec *C. neogracile* afin de voir quelles modifications physiologiques étaient mises en place en cas de compétition, et à quel niveau de compétition (plus ou moins de compétiteurs).

3.2. Article 3 - How does Pseudo-nitzschia multiseries*, a toxic diatom, compete with diatoms of the* Chaetoceros *genus?*

How does *Pseudo-nitzschia multiseries,* a toxic diatom, compete with diatoms of the *Chaetoceros* genus?

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Running head: competition between two diatoms.

Abstract

Diatoms *Pseudo-nitzschia* spp. are capable of blooming under different environmental conditions. As *Pseudo-nitzschia* spp. are not known to produce allelopathic compounds, competitive interactions with other phytoplankton are likely based upon metabolic performance. In this study, we used flow cytometry to assess the physiology of *Pseudo-nitzschia multiseries* co-cultured with different concentrations of a non-toxic and non-allelopathic diatom, *Chaetoceros neogracile*. Competition caused a decrease of the growth rate of *C. neogracile*; conversely, *P. multiseries* growth rate was not modified. Culture of *P. multiseries* reached a higher maximum cell density in co-cultures, independent of the cell density of *C. neogracile*. To maintain growth rate, *P. multiseries* modified metabolism, enhancing both primary metabolism (esterase activity) and photosynthetic capacity (chlorophyll autofluorescence), with cells exposed to higher densities of *C. neogracile* exhibiting the highest metabolic modifications. As a result, *P. multiseries* exposed to higher *C. neogracile* cell densities had higher lipid content, especially in late-log and stationary growth phases. Presence of *C. neogracile* as a competitor did not modify domoic acid production by *P. multiseries*.

Key words: diatoms, competition, *Pseudo-nitzschia*, domoic acid

Introduction

As demonstrated by Hutchinson (1961) with a phenomenon named the paradox of the plankton, single species of phytoplankton can never been found alone in the environment. Indeed, high number of phytoplanktonic species can co-exist with no exclusion, while living with some limited resources. Many factors could explain this paradox, such as symbiosis and commensalism, differential pressure of predation, or rapid environmental change (Hutchinson, 1961). Recent work has proposed that this paradox can be resolved by factors such as size-selective grazing (Wiggert et al., 2005), spatio-temporal heterogeneity (Miyazaki et al., 2006) or environmental fluctuations (Descamps-Julien and Gonzalez, 2005). More generally, some researchers suggest that ecological and environmental factors continually interact such that the planktonic habitat never reaches an equilibrium for which a single species is favoured (Scheffer et al., 2003). Very rarely, a mono-algal bloom can occur, but this never lasts very long (Millie et al., 1997). More often, when one species blooms, the other surrounding microalgae can survive and continue growing alongside the dominant species (e.g. during a *Pseudo-nitzschia* sp. bloom, Quijano-Scheggia et al., 2008). Sometimes, a bloom can be formed and composed by different species, as observed in the spring when the "diatom bloom" occurs (Suzuki et al., 2011). During blooms, nutrients are quickly removed from the water (Hayakawa et al., 1997), especially silicates (Diekmann et al., 2009), and there is competition for light (Litchman, 2003), also depending on nutrients (Floder and Burns, 2005). To survive in such an environment, algae have thus to develop different mechanisms. Allelopathy can be one of them (Legrand et al., 2003), but depends on species-specific interactions (Tillmann et al., 2007) and is thus only suitable to remove some competitive species. For non allelopathic species, being a better competitor, as well as developing specific metabolism (e.g. using less light, Yoshiyama et al., 2009) are others ways to survive, regardless of the competitor and its species (i.e. both intra- and inter-species competition).

Species of *Pseudo-nitzschia* are known to produce domoic acid (DA), a neurotoxin, but neither the cells, nor the toxin, have been shown to be allelopathic to other potentially-competing phytoplankton (Lundholm et al., 2005). Nevertheless, *Pseudo-nitzschia* species are found all around the world (reviewed by Lelong et al., 2012b) and often co-occur with many other species (Almandoz et al., 2008), including *Chaetoceros* spp. (Gailhard et al., 2002). This is often the case in France, in the coastal waters of Brittany, where *Pseudo-nitzschia* spp. cells are often observed at various concentrations co-occurring with blooms of *Chaetoceros*

spp., including *Chaetoceros socialis.* These two genera have been observed to bloom together in the spring (e.g. in Douarnenez Bay, Brittany, with co-occurrence in March and May-August 2010 at 10^5-10^6 cells l^{-1}, REPHY). Both species are thought to be competitors for the same resources. As DA has been shown to be a chelator of copper and iron (Rue and Bruland, 2001), a hypothesis may be that DA is produced by *Pseudo-nitzschia* to outcompete other algae for the acquisition of trace metals. Some non-toxic species of *Pseudo-nitzschia* can also bloom (Kaczmarska et al., 2007), suggesting that DA cannot be the only mechanism used competitively by *Pseudo-nitzschia*. Physiological adaptations or different mechanisms probably occurred depending on intra and inter-specific competition.

The aim of this study was therefore to assess the impact of competition on the physiology of *Pseudo-nitzschia* spp., analysing interactions between *P. multiseries* and *C. neogracile*, with special focus on *P. multiseries*. Cells of *P. multiseries* and *C. neogracile* were co-cultured with three different initial concentration ratios (*P. multiseries*:*C. neogracile* ratio of 1:0.5, 1:1 and 1:2), to assess how cell density can influence the outcome of competition. As mentioned previously, *Pseudo-nitzschia* species do not seem to produce any allelopathic compounds (Lundholm et al., 2005). Both species, *P. multiseries* and *C. neogracile*, were nonetheless also tested and confirmed non allelopathic (by testing effect of supernatant of *P. multiseries* on *C. neogracile* and of *C. neogracile* on *P. multiseries*, data not shown), thus only competition was examined. To assess if and how *P. multiseries* modifies its metabolism during competition, flow cytometry (FCM) was used. Compared to traditional methods where cells were only counted, FCM allowed measurements of cell physiology. Physiological measurements included the percentage of dead cells, primary metabolic activity through esterase activity, and energy storage in lipid. Measurements of DA content and bacterial density were also performed.

Material and methods

Culture conditions

Batch cultures of the marine diatoms *Pseudo-nitzschia multiseries* CCAP 1061/32 and *Chaetoceros neogracile* CCAP 1010/3 were grown in sterilized f/2 medium (Guillard and Hargraves, 1993) at 16.6°C (±1.0°C). Cultures were illuminated by cool-white bulbs (OSRAM) on a dark:light cycle of 12:12 h with an irradiance of 131 µmol photons m^{-2} s^{-1}. Cultures contained bacteria and were grown without antibiotics.

Experiment

Cultures of *P. multiseries* in mid-exponential phase were inoculated at 4200 cells ml^{-1}, in triplicate, both in mono-algal cultures or in bi-algal cultures with different cell densities of *C. neogracile*, to obtain ratios of *P. multiseries*:*C. neogracile* of 1:0.5, 1:1 and 1:2. Cultures of *C. neogracile* were inoculated at 2000, 4000 or 8000 cells ml^{-1}, both in mono-algal cultures or in bi-algal cultures. Before each sampling, cultures were homogenized by gentle manual shaking. Both algae and bacteria were counted in cultures from flow-cytometer plots. Physiological measurements (see below) were performed on *P. multiseries* cells (both in mono-algal and in bi-algal cultures) on days 3, 5, and then every day until the end of the stationary phase (between day 7 and day 19).

Specific growth rate, morphological and physiological variables

Cell count, morphological and physiological measurements, quantification of bacteria associated to *P. multiseries*, and percentage of dead bacteria in the cultures were assessed using flow cytometry according to Lelong et al. (2011). A flow cytometer (FACSCalibur BD Biosciences, San Jose, CA, USA) with a blue argon laser (488 nm) was used, with the same settings for the duration of the experiment to allow comparison between days. Cells of *P. multiseries* and *C. neogracile* were distinguished using Forward Scatter (FSC, light scattered less than 10 degrees) and Side Scatter (SSC, light scattered at a 90 degree angle).

Specific growth rate and morphological variables

Specific growth rate (μ, d^{-1}) and rate of decline (d^{-1}) were determined by linear regression of the natural log (cell density) versus time using the following formula during exponential phase and stationary phase respectively.

Growth and decline rate = $\ln(N_1-N_0)/\Delta t$ (in days).

FSC and SSC were also measured to provide light diffraction related to morphological information.

Physiological measurements

FL3 fluorescence (red fluorescence at 670 nm) was used as a relative index of cellular chlorophyll content. Mortality of *P. multiseries* was assessed by staining cultures with 0.1 µM of SYTOX Green (Molecular probes, Invitrogen, Eugene, Oregon, USA) for 30 minutes and quantifying FL1 fluorescence (stained cells were considered as dead cells). Esterase activity (an indicator of primary metabolic activity) was assessed by staining with 3 µM of fluorescein

di-acetate (FDA, Molecular probes, Invitrogen, Eugene, Oregon, USA) for 6 minutes and quantifying FL1 fluorescence. A working solution of FDA at 300 µM was prepared before each experiment. Intracellular lipid content was assessed by staining lipids with 10 µM of BODIPY 493/503 (Molecular probes, Invitrogen, Eugene, Oregon, USA) for 30 minutes and quantifying FL1 fluorescence.

Domoic acid

Domoic acid (DA) content was quantified using ASP ELISA kit (Biosense Laboratories, Bergen, Norway), following the manufacturer's protocol. Each triplicate culture was analyzed with technical duplicates. Total DA (both intracellular and dissolved) was measured on sonicated cultures, and dissolved DA was measured after filtration of fresh cultures at 0.22 µm. Intracellular DA was calculated by deducting dissolved DA from total DA.

Bacteria

Cell density and viability of free-living bacteria associated to *P. multiseries* and *C. neogracile* cultures were also assessed using flow cytometry according to Lelong et al. (2011). Briefly, bacteria were analyzed after 15 min incubation with a final concentration of 1/10000 of the commercial solution of SYBR Green I (Molecular probes, Invitrogen, Eugene, Oregon, USA) and propidium iodide (PI, Sigma, St. Louis, MO, USA) at 10 µg ml^{-1}.

Statistics

Effect of competition on the growth rate and maximal concentration of both *P. multiseries* and *C. neogracile* was tested using one way ANOVAs with the software StatGraphics Plus (Manugistics, Inc, Rockville, MD, USA). The test of rank used was the Tukey test (variance homogeneity was first tested and confirmed for all variables). To compare *P. multiseries* physiology with or without *C. neogracile*, one way ANOVAs were performed on each day and the Tukey rank test was used. For all statistical results, a probability of $p < 0.05$ was considered significant.

Results

Growth of *Chaetoceros neogracile* and *Pseudo-nitzschia multiseries*

Cultures of *C. neogracile* grew with a growth rate between 0.55 and 0.60 d^{-1} when inoculated alone (Fig 1A). Competition with *P. multiseries* caused a decrease in growth rate of *C.*

neogracile ($p < 0.01$), but no effect of the *P. multiseries*:*C. neogracile* ratio upon *C. neogracile* growth rate was found ($p > 0.05$, Fig. 1A). Decrease in growth rate of *C. neogracile* was not dependent upon inoculum size (Fig. 1B) when grown alone. When co-cultured with *P. multiseries*, *C. neogracile* decline rate was less rapid, resulting in a longer stationary phase (Fig. 1B-C). The *P. multiseries* decline rate was slower the higher the initial ratio (i.e. with higher number of *P. multiseries* per *C. neogracile* cell). Although *C. neogracile* did not grow as quickly, it reached higher maximum cell densities when grown with *P. multiseries* than when grown alone (Fig. 1C).

Presence and initial cell density of *C. neogracile* did not modify *P. multiseries* growth rate ($p > 0.05$, Fig. 1D); however, the presence of *C. neogracile* increased the maximal cell density of *P. multiseries*, regardless of the initial ratio (Fig. 1D).

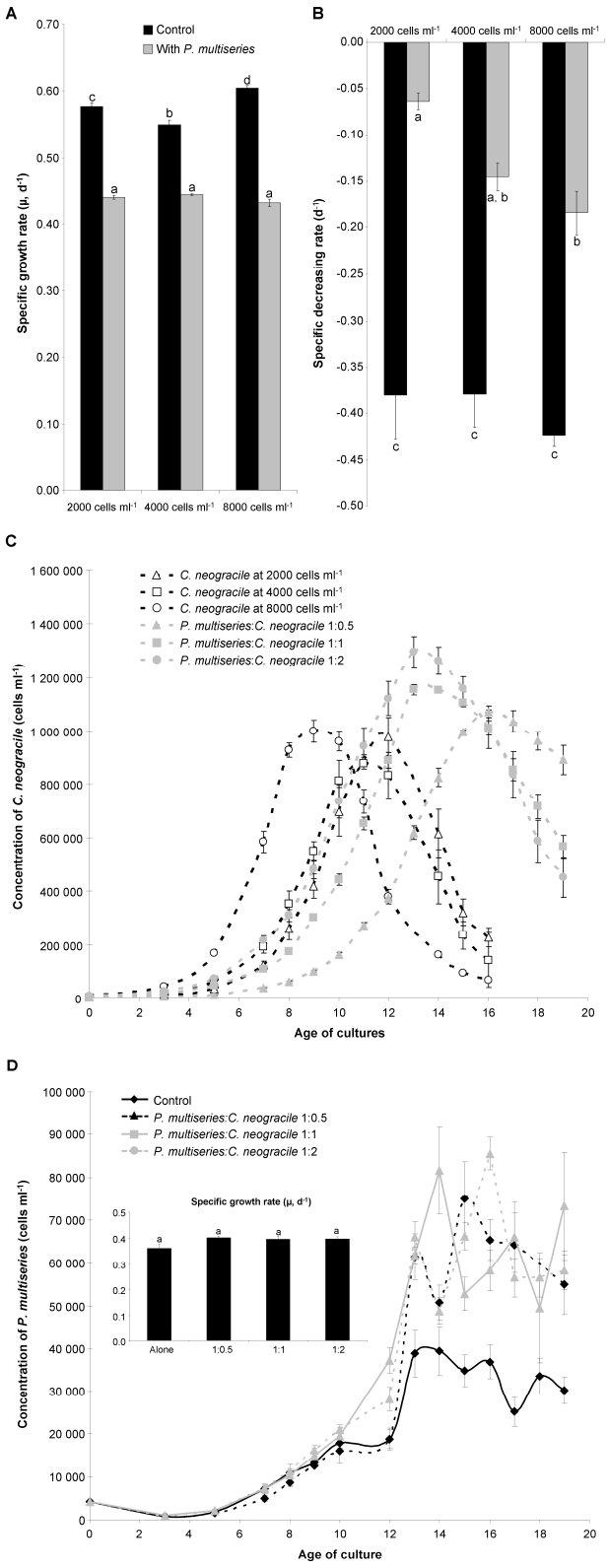

Figure 1. (A) Specific growth rate (µ, d^{-1}) and (B) decreasing rate (d^{-1}) of *C. neogracile* in control culture (black) and in co-cultures with *P. multiseries* (grey) at an initial concentration of 2000 cells ml^{-1}, 4000 cells ml^{-1} and 8000 cells ml^{-1}. (C) Concentration of *C. neogracile* in control cultures (open dark symbols) or in co-cultures (filled grey symbols), with an initial concentration of *C. neogracile* of 2000 cells ml^{-1} (ratio of 1:0.5, triangles), 4000 cells ml^{-1} (ratio of 1:1, squares) and 8000 cells ml^{-1} (ratio of 1:2, circles). (D) Concentration of *P. multiseries* in control cultures (full black line) or in co-culture with *C. neogracile* at an initial ratio of 1:0.5 (dotted black line), 1:1 (full grey line) or 1:2 (dotted grey line). In the inside box, specific growth rate (µ, d^{-1}) of *P. multiseries* for each growth curve. Mean ± SE, n = 3.

Physiology of *P. multiseries*

The percentage of dead *P. multiseries* decreased during exponential phase, as cells were growing and increased during stationary phase, regardless of the treatment (Fig. 2A). The percentage of dead algal cells increased faster and was significantly higher at day 17 for mono-algal cultures, which also reached stationary phase earlier. There were no significant differences in percentage of dead cells between *P. multiseries* cultures started at varied initial *P. multiseries*:*C. neogracile* ratios.

From day 10 to the end of the experiment, FL3 of *P. multiseries* increased with increasing initial concentration of *C. neogracile*, with significant differences between each treatment, while the FL3 of mono-algal cultures decreased (Fig. 2B).

Lipid content of cells, estimated with the BODIPY fluorescence, decreased for all cultures during exponential phase (until day 14) and increased during stationary phase (Fig. 2C). From day 14 to the end of the experiment, cultures exposed to the highest initial cell density of *C. neogracile* exhibited the highest increase in lipid content, while mono-algal cultures had the lowest lipid content (Fig. 2C).

Increasing initial concentration of *C. neogracile* induced a significant increase of FDA uptake, thus of esterase activity (Fig. 2D). Esterase activity increased after mid-exponential phase for all the cultures, but to a different extent for different experimental treatments (Fig. 2D). FDA uptake by *P. multiseries* co-cultured with *C. neogracile* at ratios of 1:1 and 1:2 were significantly higher than mono-algal cultures after day 7, but *P. multiseries* inoculated at a ratio of 1:0.5 cells of *C. neogracile* exhibited significantly higher esterase activity only after day 15 (Fig. 2D).

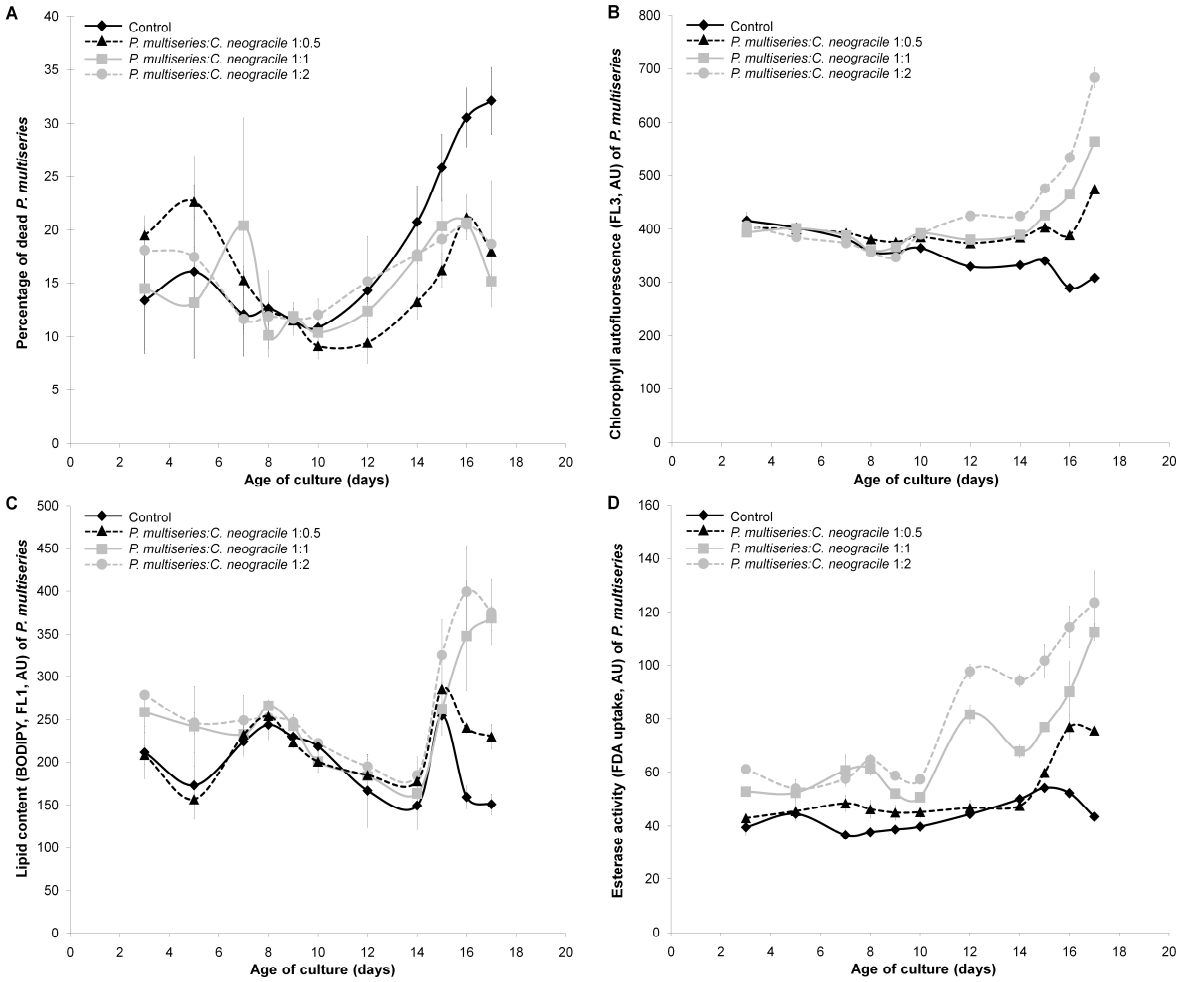

Figure 2. (A) Percentage of dead *P. multiseries* (cells permeable to SYTOX Green), (B) chlorophyll autofluorescence (FL3, in arbitrary units), (C) lipid content (BODIPY fluorescence, in arbitrary units) and (D) esterase activity (FDA uptake, FL1, in arbitrary units) of *P. multiseries* in control cultures (full black line) or in co-culture with *C. neogracile* at an initial ratio of 1:0.5 (dotted black line), 1:1 (full grey line) or 1:2 (dotted grey line). Mean ± SE, n = 3.

Domoic acid production

Total DA per cell did not vary regardless of the presence of *C. neogracile* ($p > 0.05$), and was not dependent upon *C. neogracile* initial cell density either (Fig. 3). The proportion of dissolved and cellular DA was the same for all *P. multiseries* populations and did not depend upon the presence of *C. neogracile*.

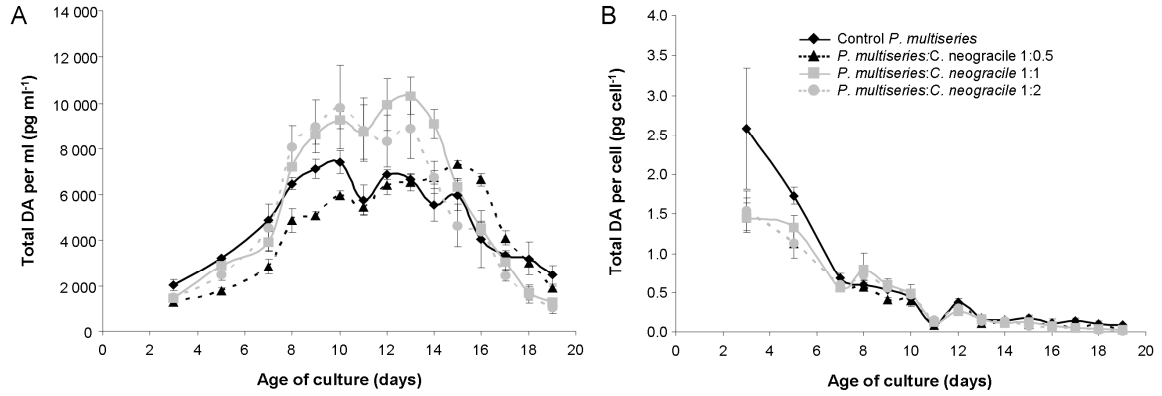

Figure 3. (A) Total domoic acid (DA) per ml of culture and (B) total DA per cell (both intra and extra cellular) of *P. multiseries* in control culture (full black line), with *C. neogracile* at an initial ratio of 1:0.5 (dotted black line), of 1:1 (full grey line) and of 1:2 (dotted grey line). Mean ± SE, n = 3.

Bacterial growth

There was significantly more bacteria in the co-cultures than in *P. multiseries* mono-algal culture ($p < 0.05$) and significantly less bacteria than in *C. neogracile* mono-cultures ($p < 0.05$), cultures with the highest initial inoculum of *C. neogracile* having the highest bacterial concentration (Fig. 4D).

The morphology of bacteria allowed clear distinction between two populations of bacteria (Fig. 4A-C), one that was almost exclusively found in *C. neogracile* cultures (population 1, Fig. 4A). A very small percentage of the bacteria were not contained in either of these two defined populations (Fig. 4A-C) and were not accounted for, as we could not attribute them to an algal species. Bacteria in population 1 (population mainly from *C. neogracile*) were less numerous in co-cultures than in *C. neogracile* cultures (Fig. 4E), with a gradient effect. Indeed, there were more bacteria in cultures inoculated with the higher cell density. Thus, bacteria from *P. multiseries* seemed to dominate over bacteria initially associated to *C. neogracile*.

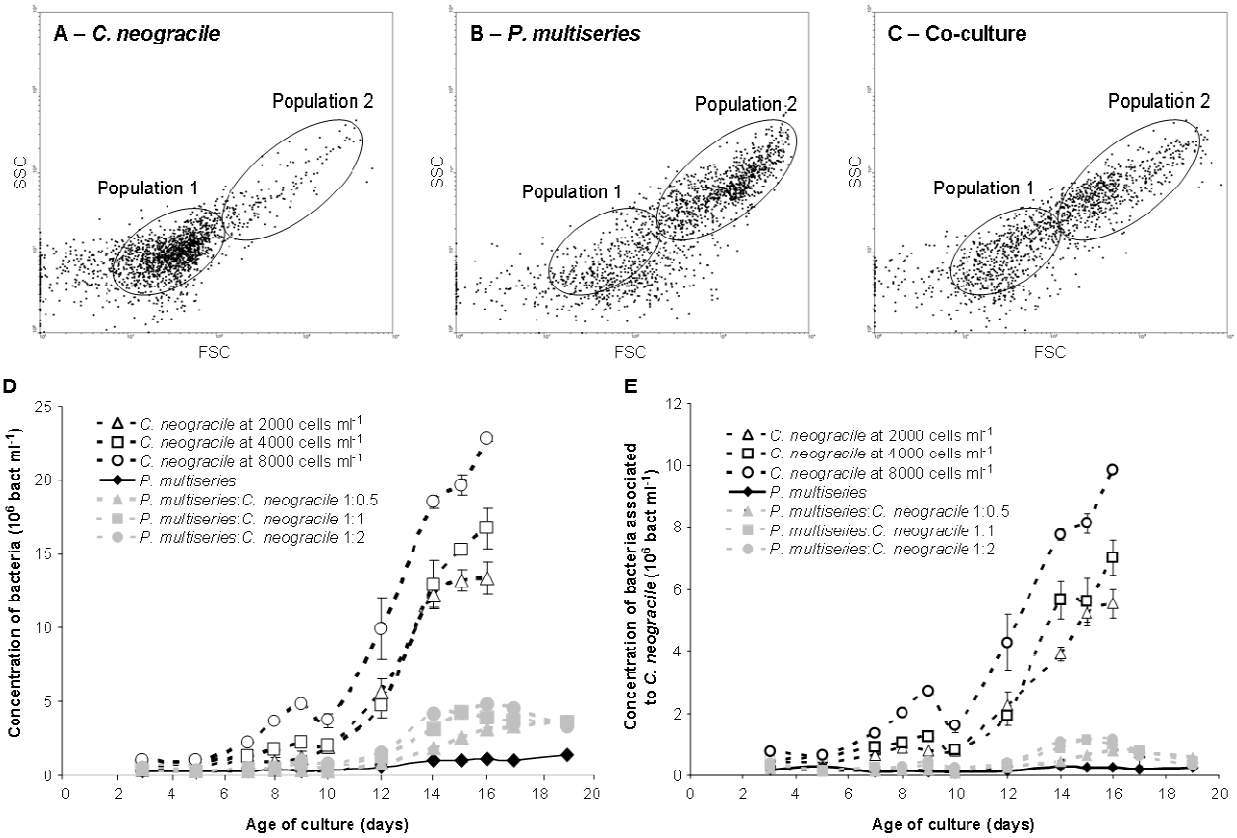

Figure 4. A-C. Cytograms of bacteria associated to (A) *C. neogracile*, (B) *P. multiseries* or (C) co-cultures depending on FSC and SSC at day 10 (initial concentration of 4000 cells ml^{-1} for *C. neogracile*). D-E. Concentration of total bacteria (D) and concentration of bacteria in population 1 (bacteria associated to *C. neogracile*, E) in control cultures (open symbols) with initial inoculation of 2000 cells ml^{-1} (open triangle), 4000 cells ml^{-1} (open square) or 8000 cells ml^{-1} (open circle), of *P. multiseries* in control culture (dark diamond), of *P. multiseries* with *C. neogracile* at an initial ratio of 1:0.5 (grey triangle), of 1:1 (grey square) and of 1:2 (grey circle). Mean ± SE, n = 3.

Discussion

This study provided evidence that *P. multiseries* up-regulates photosynthetic and primary metabolism in response to competition with a sympatric diatom, *C. neogracile*. Indeed, from mid exponential phase to the end of the experiment, chlorophyll autofluorescence of *P. multiseries*, related to chlorophyll content of cells, increased with increasing cell density of *C. neogracile*. This increase in chlorophyll autofluorescence could be a compensatory mechanism developed by *P. multiseries* to counteract cell shading by *C. neogracile*. Indeed, on day 10, when chlorophyll autofluorescence started to increase, *C. neogracile* was still in exponential-phase growth, and cultures with the highest *C. neogracile* initial inoculum also had the highest *C. neogracile* cell density. Shading can allow a species to outcompete its competitors (Pouvreau et al., 2007). Litchman and Klausmeier (2001) already demonstrated that, with increasing cell density, shading also increased, and that cells increase chlorophyll content, or at least photosynthetic performance, to acquire as much light as possible. In our

experiment, we were unable to measure QY because the two species were diatoms and thus had the same pigments and were excited at the same wavelength. This compensatory mechanism seemed to work well, as cells of *P. multiseries* grew well under competition. Even though *C. neogracile* reached the same maximal cell density, chlorophyll content of *P. multiseries* remained higher in cultures with the higher initial ratio. At this time, all the *P. multiseries* cultures were in stationary phase. Increasing chlorophyll content may be one mechanism by which *P. multiseries* maintains growth rate when co-cultured with faster-growing algae. The outcome of competition would probably change under different light conditions (Floder et al., 2002). Cells of *P. multiseries* not only enhanced photosynthetic performance, but also esterase activity from the beginning of the exponential phase until the end of the experiment. Esterase activity was enhanced to a higher extent in *P. multiseries* grown with higher numbers of competing *C. neogracile*. Cells of *C. neogracile* also exhibited increases in esterase activity when the competition was higher (i.e. the initial ratio was lower, data not shown). Nevertheless, Zhang et al. (2007) found the opposite result, with cells of *Chlorella pyrenoidosa* and *Microcystis aeruginosa* having decreased esterase activity when co-cultured. When responding to competitors, *P. multiseries* modified its metabolism, to a higher extent with higher number of competitors. These modifications include an increase in chlorophyll content and primary metabolism represented by esterases. Because of these modifications, growth rate was maintained. At the end of the exponential phase, *P. multiseries* stopped growing and started to store energy as lipid. The amount of stored lipids in *P. multiseries* was increased with increasing *C. neogracile*/*P. multiseries* ratio.

The presence of *P. multiseries* retarded the growth rate of *C. neogracile*, with no effect of the initial ratio on growth rate, thus *C. neogracile* growth lasted longer in co-cultures. An increase in *C. neogracile* maximal cell density during competition also was observed compared to *C. neogracile* culture without competitors. But once again, initial concentration (and thus initial ratio) had no effect upon maximal cell density. Similar to our results, co-cultures of *Skeletonema costatum* with *Heterosigma akashiwo* also resulted in increased maximal cell densities compared to mono-algal cultures (Xu et al., 2011). The presence of *P. multiseries* did not inhibit *C. neogracile* maximal population density, it just slowed it down (lower growth rate), suggesting that *C. neogracile* population potential was not negatively affected by *P. multiseries*, as maximal cell density was higher than in mono-culture. Co-cultures also slowed down cell decline rate; thus, the stationary phase of *C. neogracile* lasted longer when grown with *P. multiseries* than when grown alone. An effect of the *C.*

neogracile:*P. multiseries* ratio could also be observed, with cultures with the higher initial number of *P. multiseries* cells for one *C. neogracile* cell (initial ratio of 1:0.5) leading to a longer stationary phase (and a lower decline rate).

In co-cultures, bacteria of *P. multiseries* took advantage over bacteria of *C. neogracile*, leading to a much lower bacterial concentration in co-cultures than in *C. neogracile* cultures. One hypothesis is that *P. multiseries* cells could have negatively impacted bacteria of *C. neogracile*, favoring bacteria of *P. multiseries,* which therefore took advantage of this negative effect. Or bacteria from *P. multiseries* cultures could have had negative effects on bacteria associated to *C. neogracile*. Bacteria in co-cultures were thus closer to the *P. multiseries* community than the *C. neogracile* community. With lower initial ratio, there were lower amounts of bacteria. Moreover, with lower amount of bacteria, cells of *C. neogracile* survived longer (lower initial cell concentration had the lowest decline rate). These results suggest that bacteria from *C. neogracile* cultures may contribute to, or at least accelerate, *C. neogracile* death. Bacteria from the *C. neogracile* culture could also be responsible for the lowest maximal cell density found in *C. neogracile* mono-cultures compared to co-cultures. Competition for nutrients may, however, explain the differences, with bacteria associated with *C. neogracile* being better competitors than the algae. Indeed, bacteria have been proven to be better competitors compared to algae for acquisition of phosphorus (Currie and Kalff, 1984; Daufresne et al., 2008; Lovdal et al., 2007) and nitrogen (Daufresne et al., 2008). Nevertheless, interaction between algae and bacteria depends on nutrient availability and can be in the form of competition, commensalism, or mutualism (Danger et al., 2007). As bacteria seemed even more competitive at limiting nutrient concentrations (Danger et al., 2007), which is probably the case in stationary phase, the decreased concentration of bacteria can explain the longer survival of microalgal cells. Bacteria from the *C. neogracile* culture were probably outcompeted by *P. multiseries*, rather than by bacteria associated to *P. multiseries*. Measurements of nitrates and silicates are planned in the following weeks to better understand competition between both algae and bacteria.

If *C. neogracile* was able to grow, but slower, *P. multiseries* growth rate was not affected by the presence of *C. neogracile.* Similarly, growth of the green algae *C. pyrenoidosa* was not modified when co-cultured with the cyanobacteria *M. aeruginosa* (Zhang et al., 2007). Cultures of *P. multiseries* also reached a higher maximal cell density than in mono-algal cultures, regardless of the initial ratio, which had no influence on the results. In co-cultures,

there were more bacteria than in *P. multiseries* control cultures, but growth was not modified and maximal cell density was enhanced. The amount of bacteria did not seem to affect *P. multiseries* to the same extent as *C. neogracile*; thus, *C. neogracile* may be more sensitive to bacteria than *P. multiseries*.

Considering only growth curves, competition did not appear to negatively impact either of the two species, but on the contrary, seemed even favorable to the growth of both species. Bacteria may partially explain these differences, as competition within the bacterial community in these experiments clearly favored one community, which might be not if less algicidal. Results could have been different with other tested ratios, as initial ratios can modify the outcome of co-cultures (Xu et al., 2011). Culture conditions (here not limiting, at least at the beginning of the experiment) can also modify the response of an alga in competition with other species (Rodrigo et al., 2009). We can assume that these algae are used to grow among other algal species, suggesting that if there is no real effect of competition, it may be attributable to older adaptations, acquired in natural environments from years of co-habitation with many different species. Favored growth can also be attributable to the alien bacterial community, with bacteria producing siderophores, which are then used by algae to acquire iron (Soria-Dengg et al., 2001). Synergism or commensalism can also explain the better growth in co-culture compared to mono-cultures (Hutchinson, 1961). Indeed, species of *Pseudo-nitzschia* and *Chaetoceros* often reach their maximal abundance the same months in France. For example, in August 2008, *Pseudo-nitzschia* sp. and *Chaetoceros* sp. were found at 10^4-10^5 and 10^5-10^6 cells l^{-1} respectively in the Morlaix Bay (Brittany), thus both grow sympatrically, at least in France (REPHY).

DA production by cells was not different among the treatments. DA per cell decreased all along the experiment, but DA per ml of cultures increased until day 13 (end of the exponential phase), showing that cells produced DA during exponential phase, and this production ended at the beginning of stationary phase. Production of DA during exponential phase has already been shown for some species of *Pseudo-nitzschia* (Adams et al., 2000; Garrison et al., 1992) but only once for *P. multiseries* (Lelong et al., 2011), and the majority of the studies found DA production during stationary phase as well (Bates, 1998; Fehling et al., 2004; Thessen et al., 2009). Bacterial community is known to modify DA production (Bates et al., 1995; Kaczmarska et al., 2005). As the bacterial community associated to *P. multiseries* was dominant, neither the bacterial community nor competition between the two

algae for nutrient acquisition may actually explain why DA production in presence of *C. neogracile* was not modified. The maintenance of the growth rate of *P. multiseries* was not attributable to DA production, as each cell produced exactly the same amount of DA regardless of the presence and cell density of *C. neogracile*. Thus, if DA is produced to acquire Cu (Wells et al., 2005), competition with *C. neogracile* may not be strong enough to reduce *P. multiseries* access to Cu and thus enhanced DA production. It can be assumed that there was probably no competition for trace metals acquisition (between *P. multiseries* and bacteria or *P. multiseries* and *C. neogracile*).

To answer the question posed in the title, "How does *Pseudo-nitzschia multiseries*, a toxic diatom, compete with diatoms of the *Chaetoceros* genus?," cells of *P. multiseries* modified metabolism to sustain growth and were able to maintain growth rate and even reach higher cell density when co-cultured with *C. neogracile*. Modifications of metabolism included increased cellular chlorophyll content, presumably conferring higher photosynthetic efficiency, and maintenance of catabolic processes, represented by esterase activity. The response of an alga to a competitor depends upon the competitor species and the external conditions (e.g. nutrients and light, grazers) and the outcome of the competition will depend upon all these variables (Rodrigo et al., 2009). It is difficult to generalize these results to other conditions, but if *P. multiseries* was able to grow when competing with a fast-growing diatom that can reach very high population densities, we can assume that cells of *Pseudo-nitzschia* would be able to grow in a mixed phytoplankton community. In France, *Pseudo-nitzschia* spp. bloom often, and these blooms are rarely monospecific (REPHY). Even when found at lower concentrations, *Pseudo-nitzschia* co-occurs with many different species (e.g. *Chaetoceros* spp., *Guinardia delicatula*, *Skeletonema costatum*, REPHY). To bloom, algae have to compete successfully with the surrounding species, and modifying metabolism may be one way to do so. Thus, to understand how cells of *Pseudo-nitzschia* can bloom, it is important to measure their metabolism, and not only production of DA. Indeed, DA did not seem implicated in the competition outcome, which explains why non-toxic *Pseudo-nitzschia* species can also bloom (Kaczmarska et al., 2007).

Acknowledgements

This work was supported by the French ministry of research (MENRT grant), Region Bretagne grant, SAD Volet 1, PSEUDOPE to HH and CNRS EC2CO Microbien to PS. We

would also like to thank Dr Gary Wikfors for his constructive comments on the manuscript and English corrections.

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3.3. Conclusion

La compétition entre *P. multiseries* et *C. neogracile* n'affecte pas les deux espèces phytoplanctoniques de la même façon. Ainsi, quand la croissance de *P. multiseries* n'est pas modifiée par la présence de *C. neogracile*, la croissance de cette dernière est plus lente en co-culture. Elles atteignent néanmoins toutes deux une concentration maximale plus élevée en co-cultures, ce qui peut laisser penser que la co-culture est un avantage, ou alors que les cellules sont adaptées à la présence d'au moins une autre espèce. Pour soutenir ce taux de croissance, *P. multiseries* modifie son métabolisme, en augmentant l'activité de ses estérases et sa capacité photosynthétique en phase exponentielle, de façon proportionnelle au nombre de cellules de *C. neogracile*. La conséquence est un stockage de lipides de réserve plus important en phase stationnaire, également proportionnel au nombre de *C. neogracile* en co-cultures. La production d'acide domoïque par *P. multiseries* n'est par contre pas affectée par la présence de compétiteurs. Les bactéries présentes dans les co-cultures sont majoritairement des bactéries associées à *P. multiseries*, qui semblent prendre le dessus sur les bactéries associées à *C. neogracile*. Ces modifications de populations bactériennes peuvent expliquer la plus forte concentration atteinte par *C. neogracile* ainsi que le déclin plus lent en co-cultures, les bactéries associées à *C. neogracile* ayant *a priori* un effet négatif sur les cellules de *C. neogracile*. Ce n'est donc pas simplement la compétition pour les nutriments (macro ou micronutriments) qui induit la production d'acide domoïque, en tout cas en conditions non-limitantes au départ.

La compétition avec une diatomée n'induit pas la production d'acide domoïque. Pourtant les cellules modifient leur physiologie et s'adaptent à cette nouvelle contrainte. Si la compétition avec une diatomée n'induit pas la production d'acide domoïque, pourquoi est-ce que la présence de bactéries est indispensable à cette production (Bates et al., 1995) ? Est-ce un mécanisme de défense spécifique contre les procaryotes ?

INTERACTIONS ENTRE *PSEUDO-NITZSCHIA* SPP. ET DIFFERENTES COMMUNAUTES BACTERIENNES

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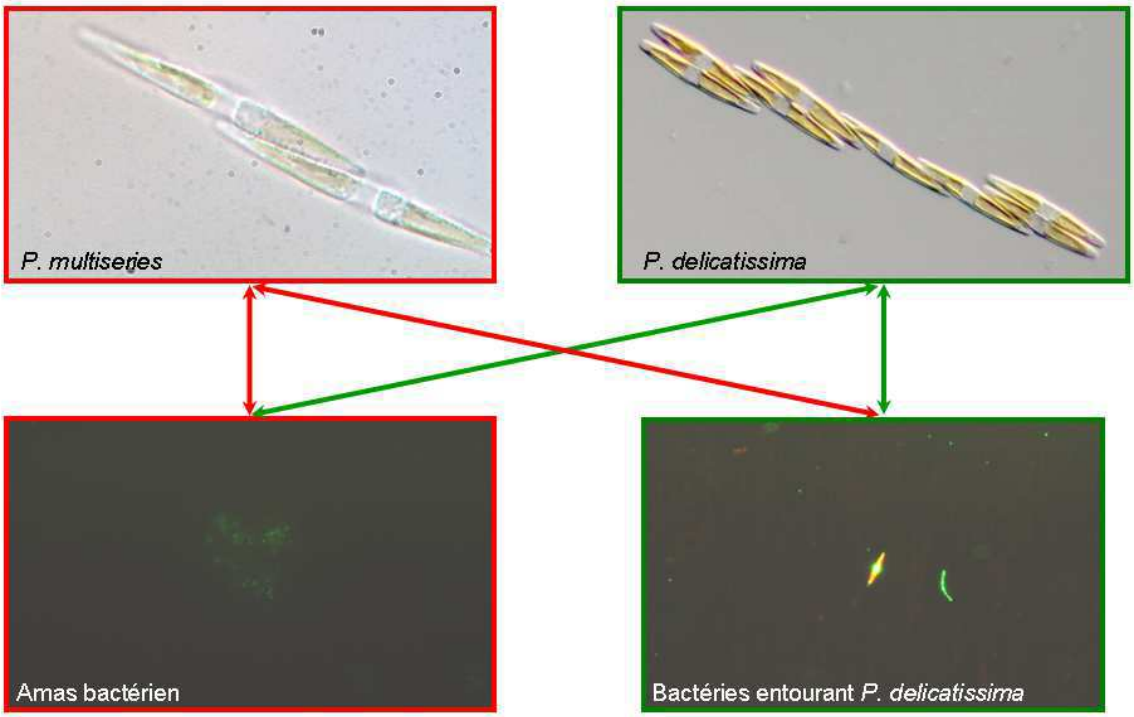

Article :

Lelong A., Hégaret H., Soudant P. Modification of domoic acid production and cell physiology after exchange of bacterial community between toxic *Pseudo-nitzschia multiseries* and non toxic *Pseudo-nitzschia delicatissima*. En préparation pour soumission à Harmful Algae.

4.1. Préambule

Microalgues et bactéries marines interagissent de manière complexe. Certaines bactéries inhibent la croissance microalgale (Wang et al., 2010, Su et al., 2011), tandis que d'autres bactéries sont indispensables à cette croissance (Shen et al., 2011), d'autres enfin n'ont aucun impact notable. Certaines bactéries vont de plus être capables d'inhiber une espèce de microalgue, ne rien faire à une deuxième et être nécessaires à la troisième (Brussaard and Riegman, 1998). Par ailleurs, certaines bactéries ne peuvent croître sans microalgues et l'apport de carbone qu'elles représentent (Fouilland and Mostajir, 2010). Il est donc impossible de dresser un simple schéma d'interaction microalgues-bactéries et les études doivent encore être menées au cas par cas. En ce qui concerne *Pseudo-nitzschia*, une partie des bactéries environnantes est indispensable, non seulement à la croissance, mais surtout à la production d'acide domoïque (Bates et al., 1995). Il a en effet été mis en évidence que des cultures rendues axéniques ne produisent plus, ou presque plus, d'acide domoïque et que cette production est restaurée dès que les bactéries sont rajoutées à la culture phytoplanctonique (Bates et al., 1995). L'ajout de différentes communautés bactériennes peut également faire varier la production d'acide domoïque (Kaczmarska et al., 2005). Si les bactéries seules ne sont pas capables de produire de l'acide domoïque (Bates et al., 2004), certaines espèces peuvent en revanche le dégrader (Stewart et al., 1998).

Pour essayer de mieux comprendre ces interactions, les deux espèces de *Pseudo-nitzschia*, une toxique et une non-toxique, ont été dans un premier temps rendues axéniques par l'utilisation d'antibiotiques. A ces cultures axéniques ont ensuite été rajoutées soit leurs propres bactéries, soit la communauté bactérienne de l'autre espèce de *Pseudo-nitzschia*. Leur croissance a ainsi pu être comparée sans bactérie, avec leurs bactéries, ou avec les bactéries associées à l'autre espèce. Le suivi de la physiologie et la production d'acide domoïque de *Pseudo-nitzschia* a ensuite été réalisé, en parallèle du suivi des communautés bactériennes, afin de caractériser les effets des bactéries sur les deux espèces de *Pseudo-nitzschia* et d'essayer d'établir un lien potentiel entre la production d'acide domoïque et l'état physiologique des cellules.

4.2. Article 4 - Modification of domoic acid production and cell physiology after exchange of bacterial community between toxic Pseudo-nitzschia multiseries *and non toxic* Pseudo-nitzschia delicatissima

Modification of domoic acid production and cell physiology after exchange of bacterial community between toxic *Pseudo-nitzschia multiseries* and non toxic *Pseudo-nitzschia delicatissima*

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Abstract

Bacteria are known to influence domoic acid production by *Pseudo-nitzschia* spp. but non toxic species have never been studied so far. In this study we aimed to compare a toxic *P. multiseries* to a non-toxic *P. delicatissima*, both in terms of DA production and physiological parameters. Bacterial communities from both species were eliminated before algae could be grown with the bacterial community of the other species. Growth of *P. delicatissima* was not affected by bacteria while *P. multiseries* grew faster without bacteria and did not grow with alien bacteria. The exchange of bacterial community resulted in an increase of the DA production by *P. multiseries* with alien species while axenic cultures did not produce any DA. Physiology of *P. multiseries* was also affected, mainly when algal were grown with alien bacteria, as a decrease of chlorophyll content and photosynthetic efficiency together with an increase of the esterase activity could be observed. On the opposite, physiology of *P. delicatissima* was barely modified by alien bacteria. The main difference was the absence of stationary phase and death of *P. delicatissima* right after the end of exponential phase.

Key words: bacteria, domoic acid, physiology, *Pseudo-nitzschia*

Introduction

In the oceans, phytoplankton is not the main living organism. Indeed, there is between 10^4 and 10^6 bacteria per ml of sea water (He et al., 2009; Seuront et al., 2010), which is much more than phytoplankton (except during blooms). Species of phytoplankton and bacteria live together and interfere in many ways. First of all they can compete each other for the acquisition of inorganic nutrients (Hitchcock et al., 2010; Maranon et al., 2010) and phytoplankton can be one source of carbon for bacteria (Fouilland and Mostajir, 2010), conversely bacteria can also be a source of carbon for mixotrophic phytoplankton (Legrand et al., 2001; Schmidtke et al., 2006). Nevertheless, interactions between phytoplankton and heterotrophic bacteria are not that simple. Indeed, some bacteria exhibit algicidal properties (Su et al., 2011; Wang et al., 2010) or induce the formation of cyst of *Lingulodinium polyedrum* for example (Mayali et al., 2007). On the other hand, some microalgae can decrease bacterial abundance or even exhibit bactericidal activities (Sanchez-Saavedra et al., 2010). Sometimes, bacteria are also necessary to microalgae (Shen et al., 2011) and can enhance their growth (Ferrier et al., 2002). Bacteria can even protect microalgal cells from allelopathic compounds (Bauer et al., 2010).In most of the cases, the interaction between microalgae and phytoplankton depends on the environmental conditions (Brussaard and Riegman, 1998).

Bacteria can also play a role in toxin production. It has thus been shown that axenic cultures of *Pseudo-nitzschia multiseries* produced no or really low levels of domoic acid (DA) and that the reintroduction of bacteria enhanced this production by 2 to 95-fold (Bates et al., 1995; Kobayashi et al., 2009; Stewart, 2008; Stewart et al., 1997). All the bacterial communities, even from *Chaetoceros* sp. cultures, enhanced production of DA when cultured with toxic *Pseudo-nitzschia* species (Bates et al., 1995). It was thus hypothesized that DA production was induced by bacteria in a non-specific way. Some bacteria seemed to be able to produce gluconolactone/gluconic acid, a sequestering agent. One hypothesis is that DA would be produce by *Pseudo-nitzschia* to compete bacteria as a "chemical scavenger" to counteract nutrient stress (Osada and Stewart, 1997; Stewart, 2008). Some species of *Pseudo-nitzschia* do not seem able to produce DA, thus how can these species outcompete bacteria? We tried to exchange the free-living bacterial community of a toxic strain of *P. multiseries* with a non-toxic strain of *Pseudo-nitzschia delicatissima*. Axenic and xenic cultures were then compared, not only in term of DA production but also in term of physiological state. The physiology and

DA production were followed along the growth, with measurement of esterase activity, lipid content, chlorophyll autofluorescence, photosynthetic efficiency and cell morphological parameters on living cells. Bacteria were counted and their survival was also followed. All these measurements allowed us to find out how cells react to different bacterial community and how DA production and physiology were modified depending on added bacterial community.

Material and methods

2.1. Culture conditions

Two species of *Pseudo-nitzschia* were used: *P. multiseries* (Hasle) Hasle (strain CLNN16, isolated from the Bay of Fundy, Canada) and *P. delicatissima* (Cleve) Heiden (strain Pd08RB, isolated from Brittany, France). They were cultured in sterilized f/2 media (Guillard and Hargraves, 1993), at 15.6°C, with an irradiance of 131 µmol photons m^{-2} s^{-1} and a dark:light period of 12:12 h. Cultures were initially xenic and grown without antibiotics. Before each sampling, cultures were gently homogenized.

2.2. Removal of bacteria

Before experiment, each culture of *P. multiseries* and *P. delicatissima* was divided into two different cultures, one treated with antibiotics (AB) and one untreated. The culture of *P. delicatissima* was much more resistant to AB than *P. multiseries*, it was thus exposed to 2:1 mg ml^{-1} of penicillin: streptomycin (P:S) during 2 d, whereas *P. multiseries* was exposed to 0.2:0.1 mg ml^{-1} of P:S and 0.02 mg ml^{-1} of ampicillin during 4 d (higher concentrations of AB killed both cells and bacteria). At the end of the treatment with AB, cultures were washed by centrifugation (10 min, 16°C, 800 g), the pellet was resuspended into new f/2 media to remove all AB. Cultures exposed to AB were not fully axenic and a few bacteria remained within the culture, but at a 10-fold lower concentration than untreated cultures.

2.3. Experiment

Experiment started two days after the end of the AB treatment, when cells were in exponential growth phase. For each species, cells were cultured in triplicates under three conditions: (i) the AB treated culture (almost axenic), (ii) the AB treated culture + bacteria of *P. multiseries* and (iii) the AB treated culture + bacteria of *P. delicatissima*, each in triplicate (Table 1). Free-living bacteria were obtained after centrifugation (10 min, 16°C, 800 g) of the untreated

cultures in the supernatant. Bacteria in these supernatants were counted using a flow cytometer and it was made sure that there were no remaining microalgal cells. Cultures of *P. multiseries* were inoculated with 4000 cells ml^{-1} of treated *P. multiseries* (i) alone (almost axenic, M0), (ii) with ~200 000 bacteria ml^{-1} of untreated *P. multiseries* (MM) or (iii) with ~200 000 bacteria ml^{-1} of untreated *P. delicatissima* (MD). Cultures of *P. delicatissima* were inoculated with 2500 cells ml^{-1} of treated *P. delicatissima* (i) alone (D0), (ii) with ~100 000 bacteria ml^{-1} of untreated *P. delicatissima* (DD) or (iii) with ~100 000 bacteria ml^{-1} of untreated *P. multiseries* (DM). Bacterial concentration was chosen to remain consistent with the bacteria/*Pseudo-nitzschia* ratio of the untreated cultures of each species. Samples were taken almost every day to count cells and performed physiological measurements.

2.4. Morphological and physiological measurements

Cell concentrations, morphological and physiological measurements, quantification of bacteria associated to *Pseudo-nitzschia* species and percentage of dead bacteria in the culture were assessed using flow cytometry according to Lelong et al. (2011). The physiological measurements were realized with a flow cytometer FACScalibur (BD Biosciences, San Jose, CA USA) with an argon blue laser (488 nm). To allow comparison between days, the same settings of the instruments were used for all the duration of the experiment. Cells of *Pseudo-nitzschia* were detected by their FL3 fluorescence. As all samples were run for 45 seconds, their concentrations were calculated from a flow-rate measurement of the flow cytometer performed each day of experiment (Marie et al., 1999). Specific growth rate (μ, d^{-1}) was determined by linear regression of the natural log(cell concentration) versus time.

Algal concentration was measured without any staining, whereas free-living bacterial concentration and percentage of dead bacteria were obtained after a staining of cultures with a final concentration of 1/10000 of the commercial solution of SYBR Green I (Molecular probes, Invitrogen, Eugene, Oregon, USA) and 10 $\mu g\, ml^{-1}$ of propidium iodide (PI, Sigma, St. Louis, MO, USA) for 15 minutes. The dead bacteria were not considered in the measurement of the growth rate of bacteria. The percentage of dead algal cells was assessed after a staining of 30 minutes of cultures with 0.1 µM of SYTOX Green (Molecular probes, Invitrogen, Eugene, Oregon, USA). Intracellular lipid content of algal cells was assessed by staining lipids with 10 µM of BODIPY 493/503 (Molecular probes, Invitrogen, Eugene, Oregon, USA) for 30 minutes and activity of primary metabolism of algal cells was assessed after staining with 3 µM of fluorescein di-acetate (FDA, Molecular probes, Invitrogen, Eugene,

Oregon, USA) for 6 minutes. A work solution of FDA at 300 µM was freshly prepared before each experiment.

Quantum yield ($QY=(F_m-F_0)/F_m$) of *P. multiseries* and *P. delicatissima* were measured using the AquaPen-C AP-C 100 fluorometer (Photo Systems Instruments, Czech Republic). QY is a measurement of the efficiency of the photosynthesis, where F_0 and F_m are respectively the minimum and maximum fluorescence of cells at 455 nm. The measurement of QY was performed after 20 min of dark adaptation of the cells at 16°C.

Domoic acid (DA) content was quantified using ASP ELISA kit (Biosense Laboratories, Bergen, Norway), following the constructor protocol. Each triplicate of culture was analyzed with technical duplicates. Cultures (cells+supernatant) were sonicated for 2 minutes to quantify total DA. Cultures were also filtered at 0.22 µm (acetate cellulose filters, Minisart, Sartorius, Göttingen, Germany) during the experiments to measure dissolved DA. Intracellular DA was obtained by the following calculation: $DA_i = DA_t-DA_d$, where DA_i=intracellular DA, DA_t= total DA and DA_d=dissolved DA.

2.5. Statistics

Results were analyzed statistically with multifactor ANOVA, to test the effect of the treatment (bacteria or not). One-way ANOVA were used to find out after which day, treatments started to have a significant effect on the parameter. The test of rank used was the LSD test. For all statistical results, a probability of $p < 0.05$ was considered significant. Statistical analyses were performed using StatGraphics Plus (Manugistics, Inc, Rockville, MD, USA).

Results

3.1. Algae growth and death

Cells of *P. multiseries* exhibited a different growth rate depending on the treatment (Fig. 1A). Cultures with no added bacteria (M0) grew faster than cultures with *P. multiseries* bacteria (MM, $p < 0.05$), while cultures with *P. delicatissima* bacteria (MD) did not grow (Fig. 1A). Cultures also reached significantly different cell density, with the fastest culture (M0) reaching the highest concentration (Fig. 1A). On the opposite, cultures of *P. delicatissima* exhibited non significant growth rate difference ($p > 0.05$), regardless of the treatment (Fig.

1B). However, cultures D0 reached a higher cell concentration and maintained it for a longer time than cultures DD ($p < 0.05$, Fig. 1B). Cultures DM, reached the lowest maximum cell concentration, did not exhibit any stationary phase and concentration decreased right after exponential phase.

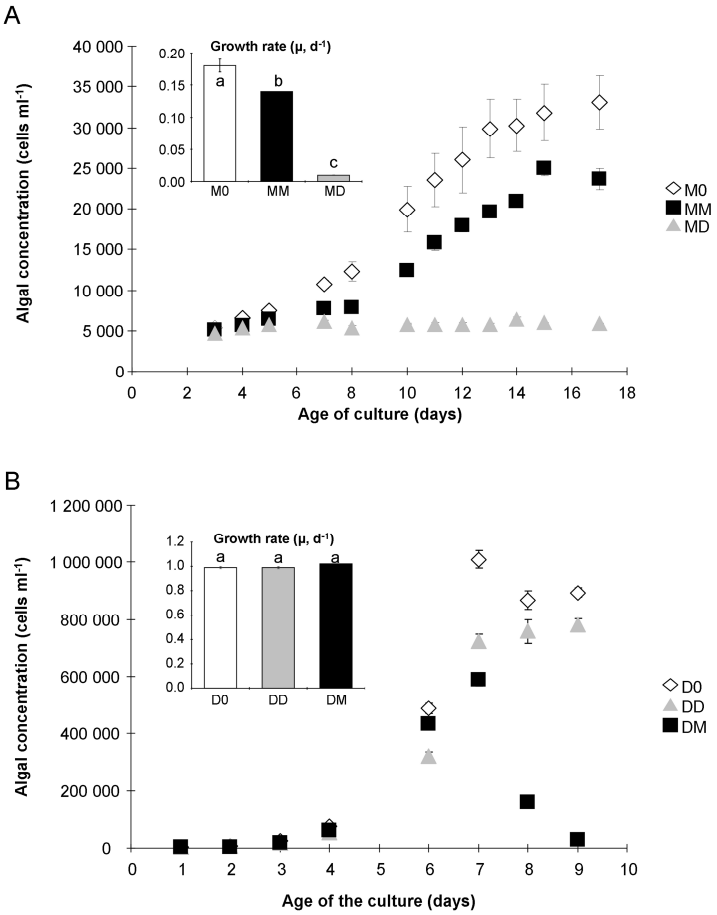

Figure 1: Concentration (cell ml^{-1}) of *P. multiseries* (A) and *P. delicatissima* (B) without bacteria (M0 and D0, open diamonds), with bacterial community of *P. multiseries* (MM and DM, filled squares) or with bacterial community of *P. delicatissima* (MD and DD, grey triangles). In each insert are found the specific growth rate (µ, d^{-1}) of both species. Letters indicate significantly different values ($p < 0.05$). Mean ± SE, n = 3.

The percentage of *P. multiseries* cells stained by SYTOX green (permeable cells, considered as dead cells) was high and almost the same for the three treatments until day 7 ($p > 0.05$, Fig. 2A). After day 7, cultures MM and M0 recovered, thus having a low percentage of dead cells, whereas almost 50% of the cells in the MD cultures were dead (Fig. 2A). All the *P. delicatissima* cultures had a percentage of dead cells decreasing between day 1 and day 6 (Fig. 2B). After day 7, DM cultures started to die faster than DD, while D0 exhibited the lowest percentage of dead cells (Fig. 2B).

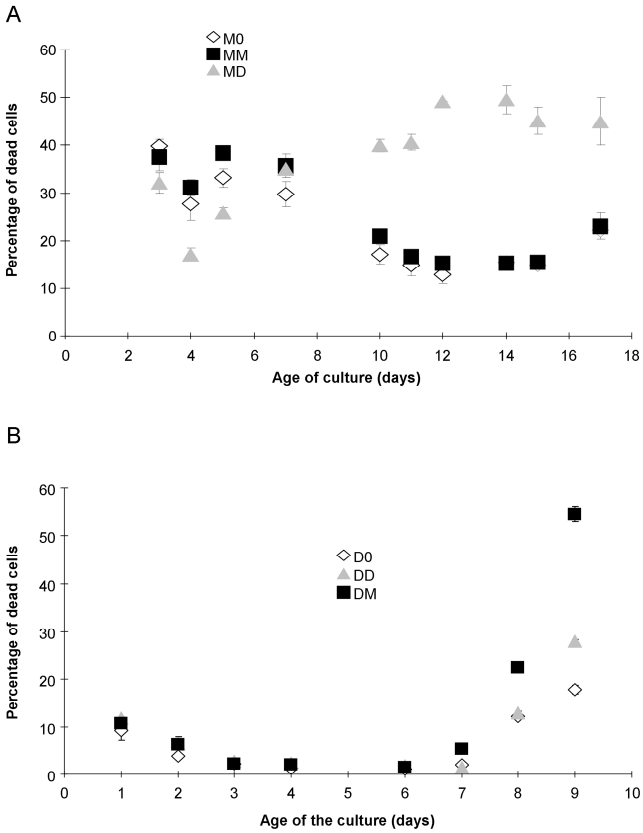

Figure 2: Percentage of dead *P. multiseries* (A) and *P. delicatissima* (B) after SYTOX green staining, without bacteria (open diamonds), with bacterial community of *P. multiseries* (filled squares) or with bacterial community of *P. delicatissima* (grey triangles). Mean ± SE, n = 3.

While SYTOX Green stains permeable cells (considered as dead), FDA stains only cells with active esterase (considered as living). As AB can compromise cell membranes, without killing them, the percentage of active cells was also measured. The percentage of active *P. multiseries* cells increased between day 3 and 4 for all cultures and remained identical until day 7 (Table 1). From day 7, the percentage of active MD cells decreased and was lower than M0 and MM cells (Table 1). The percentage of active *P. delicatissima* cells was the same regardless of the treatment until the last day, where DM had less active cells than DD, while D0 had the highest percentage of active cells (Table 2).

3.2. Algae morphology

FSC and SSC values of *P. multiseries* were different according to the treatment. Indeed, MD cultures had lower FSC and SSC than M0, and M0 had lower FSC and SSC values than MM (Table 1). FSC and SSC of *P. delicatissima* D0 were significantly lower than DD and DM cultures whereas DM and DD had significantly the same morphological parameters (Table 2).

3.3. Bacterial growth and morphology

Only free-living bacteria were counted, with all the bacteria exhibiting two growth phase. Bacteria grew slower during the first growth phase, which lasted until day 8 for *P. multiseries* and day 4 for *P. delicatissima*. M0 cultures had 4-fold less bacteria than MM and MD had the same bacterial concentration than MM. Bacteria of M0 cultures grew faster ($\mu = 0.16 \pm 0.02$ d^{-1} and 0.39 ± 0.02 d^{-1}) and reached MD and MM bacterial concentrations at day 12 (Fig. 3A). Bacteria of MM grew at 0.15 ± 0.01 d^{-1} and 0.20 ± 0.01 d^{-1} while bacteria of MD grew at 0.14 ± 0.01 d^{-1} and 0.23 ± 0.00 d^{-1}. D0 culture of *P. delicatissima* had almost no bacteria until day 6 and bacteria started to grow between day 6 and 9 with a growth rate of 1.83 ± 0.52 d^{-1} (Fig. 3B). On the opposite, DD bacteria grew at 0.40 ± 0.04 d^{-1} and 0.57 ± 0.02 d^{-1} and DM bacteria grew at 0.30 ± 0.00 d^{-1} and 0.67 ± 0.00 d^{-1} during the two growth phases respectively, with significantly more bacteria in the DM culture (Fig. 3B).

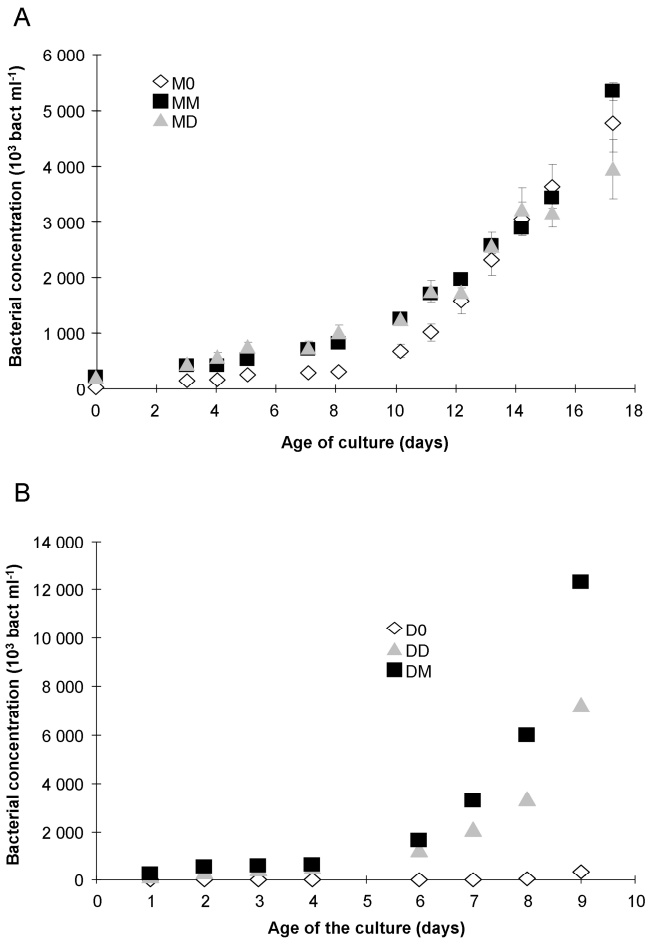

Figure 3: Concentration of free-living bacteria (bact ml^{-1}) associated to *P. multiseries* (A) and *P. delicatissima* (B) after Propidium Iodide and SYBR Green staining. Cultures were grown without bacteria (open diamonds), with bacterial community of *P. multiseries* (filled squares) or with bacterial community of *P. delicatissima* (grey triangles). Mean ± SE, n = 3.

The morphology (FSC and SSC) of each bacterial community was different (Table 3). In the cultures of *P. multiseries*, FSC and SSC parameters were similar between M0 and MM cultures and increased along the experiment. FSC of bacteria associated to *P. multiseries* in MD cultures was lower than in MM and M0 and distinguishable from bacteria associated to *P. delicatissima* which exhibited a significantly higher SSC. On the opposite, FSC and SSC of bacteria growing in *P. delicatissima* decreased in the three treatments. Morphology of bacteria associated to *P. delicatissima* in D0 and DD cultures was the same at the end of the experiment (when bacteria were detectable), while bacteria initially associated to *P. multiseries* in DM cultures were different from *P. delicatissima* original bacterial community. There were no bacteria from the original bacterial community associated to *P. delicatissima* in DM cultures, only the bacteria associated to *P. multiseries* that we added. Morphology related parameters of bacterial community associated to *P. multiseries* or *P. delicatissima* was modified when they were grown with *P. multiseries* or *P. delicatissima* (Table 3).

3.4. Domoic acid

M0 cultures exhibited almost no DA per ml of cultures, indicating no production of DA (Fig. 4A). On the opposite, M0 and MM cultures produced DA. Indeed concentration of total DA per ml culture increased until day 15 to 3444 ± 297 pg ml^{-1} for MD cultures and until the end to 1102 ± 212 pg ml^{-1} for MM cultures (Fig 4A). M0 cultures exhibited less than 0.01 pg $cell^{-1}$ of total DA, regardless of the growth phase, thus dissolved and intracellular amount of DA were not measured (Fig. 4B). MD cultures had significantly more DA than M0 and MM cultures and exhibited a different pattern of excretion (Fig. 4B). MM cultures had 0.10 pg $cell^{-1}$ of total of DA on day 3 and this concentration decreased all along the growth curve to 3-fold less DA (Fig. 4B).

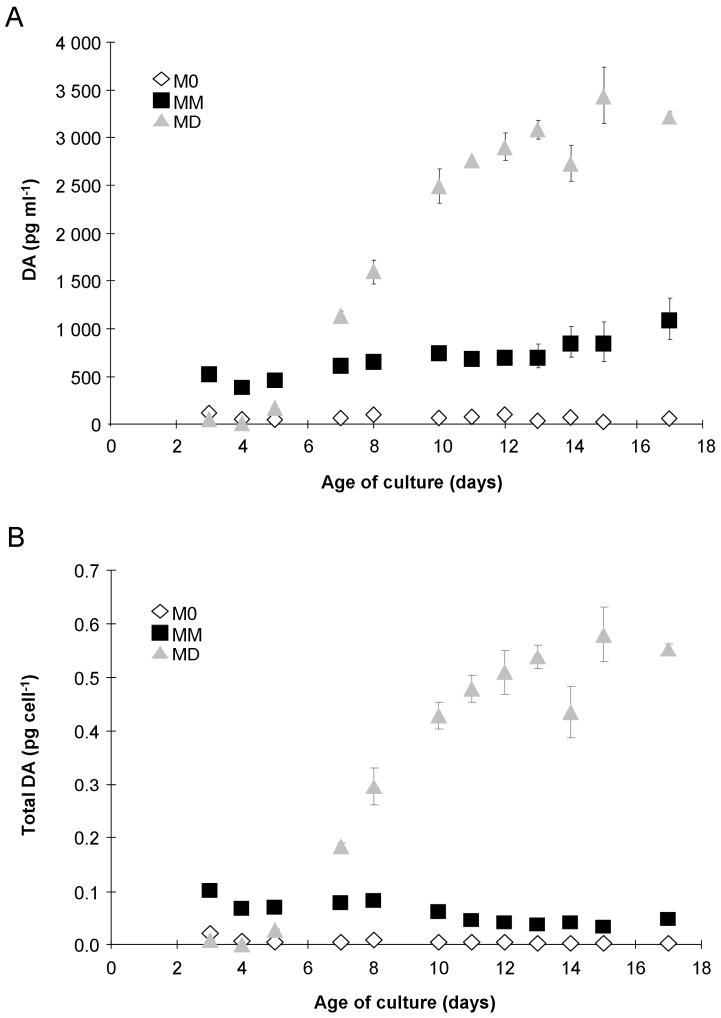

Figure 4: A-B. Total domoic acid (DA) in pg ml^{-1} (A) or pg cell^{-1} (B) in *P. multiseries* cultures grown without bacteria (open diamonds), with bacterial community of *P. multiseries* (filled squares) or with bacterial community of *P. delicatissima* (grey triangles).

Almost all the DA contained in the MM cultures was found dissolved (Fig. 5A). Cells were not growing but started to produce DA. DA production per cell increased to ~6-fold more than the concentration found in MM cultures. Until day 11, almost all the DA was stored inside the cells (> 80 %) and after day 11, cells started to release DA in the medium to reach around 50 % of the total DA (Fig. 5B). Cultures of *P. delicatissima* did not exhibit any dissolved or intracellular DA, regardless of the treatment and growth phase.

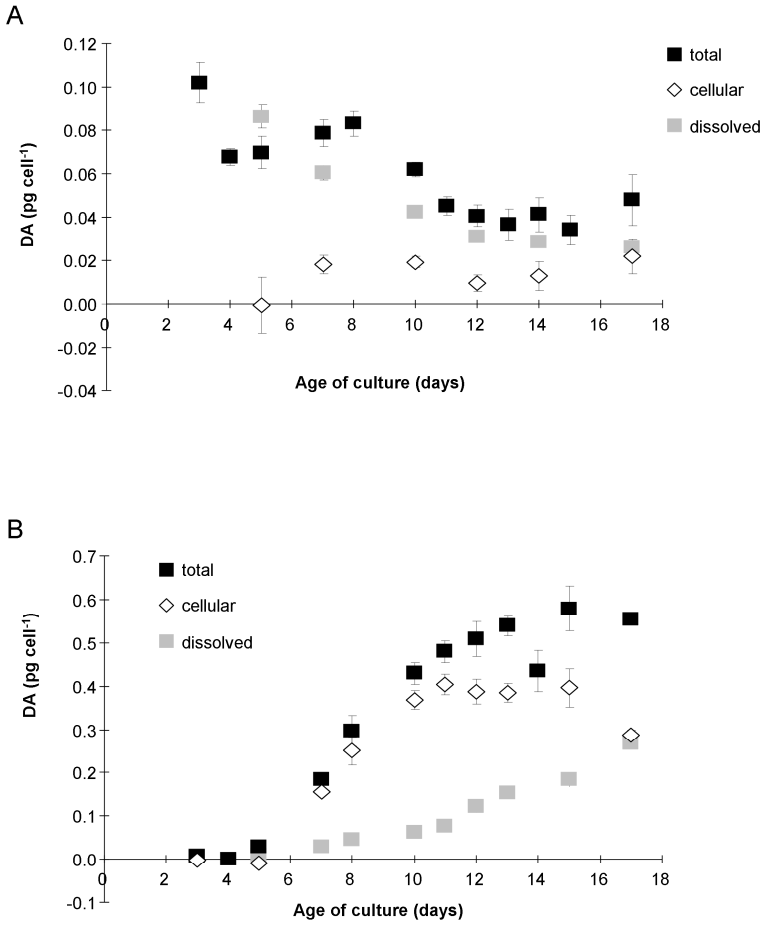

Figure 5: Total DA (filled squares), cellular DA (open diamonds) and dissolved DA (grey squares) of *P. multiseries* grown with their bacteria (A) or with *P. delicatissima* bacteria (B). Mean ± SE, n = 3.

3.5. Photosynthetic parameters

Cell autofluorescence decreased for MD cultures as cultures were not growing (Table 1). FL3 values of M0 and MM cultures were similar and increased during exponential phase, to reach significantly higher values than MD cultures after day 8 (Table 1). Cultures M0 and MM had the same values of QY along the experiment ($p > 0.05$), with a decrease after day 10 and till the end of the experiment, linked with the second part of the exponential growth phase. Cultures of MD had a lower QY than M0 and MM until day 12 and they exhibited an increase of their QY value from day 12 to day 15, with higher values than MM and M0 cultures (Table 1). All the cultures of *P. delicatissima* exhibited an increase of cell autofluorescence together with exponential growth phase, except for days 2 to 4, where D0 had a lower autofluorescence than DD and DM (Table 2). QY of *P. delicatissima* (D0, DD and DM) increased during the early exponential phase, and was similar regardless of the treatments.

During the decrease of the QY, D0 cultures had a slightly but significantly lower QY than DD and DM, all following the same pattern (Table 2).

3.6. Esterase activity and lipid content

The uptake of FDA was significantly lower for M0 cultures than MM cultures but they exhibited the same pattern of activity. On the opposite, MD cultures had different pattern of activity, with an increased activity during the first days of growth (Table 1). The three cultures D0, DD and DM had the same peak of uptake at day 2. During stationary phase, when D0 cultures were dying, they had a significantly higher uptake of FDA than DD and DM cultures, thus a higher esterase activity (Table 2).

The BODIPY fluorescence of *P. multiseries* cells (i.e. their lipid content) followed the same pattern regardless of the treatment, with a decrease of lipid content during growth (even for M0 that did not grow). Lipid content of MM cultures was higher than M0 and MD cultures; while M0 and MD cultures were not significantly different (Table 1). The lipid content of *P. delicatissima* exhibited a totally different pattern, as lipid content increased in cells during exponential phase and decreased at the beginning of stationary phase, to finally increase during stationary phase, regardless of the treatment (Table 1). D0 cultures had significantly less lipids than DM and DD cultures (Table 2).

3.7. Principal component analysis

MM and M0 cultures are quite close, regardless of the day and their physiological parameters varied with the same pattern (Fig. 6A). On the opposite, MD cultures did not evolve like other *P. multiseries* cultures and were mainly characterized by high DA content and high ratio bacteria per *Pseudo-nitzschia* cells and lower morphological (FSC and SSC) and physiological FL3 and BODIPY parameters compared to MM and M0. DD and DM cultures were identical between day 1 and day 4 and then evolved with the same pattern but with a delay of one day (Fig. 6B). D0 cultures were different from the other cultures and were mainly characterized by a high percentage of dead bacteria, a higher concentration of *P. delicatissima* and lower values of morphological (FSC) and physiological parameters (BODIPY, FDA, FL3).

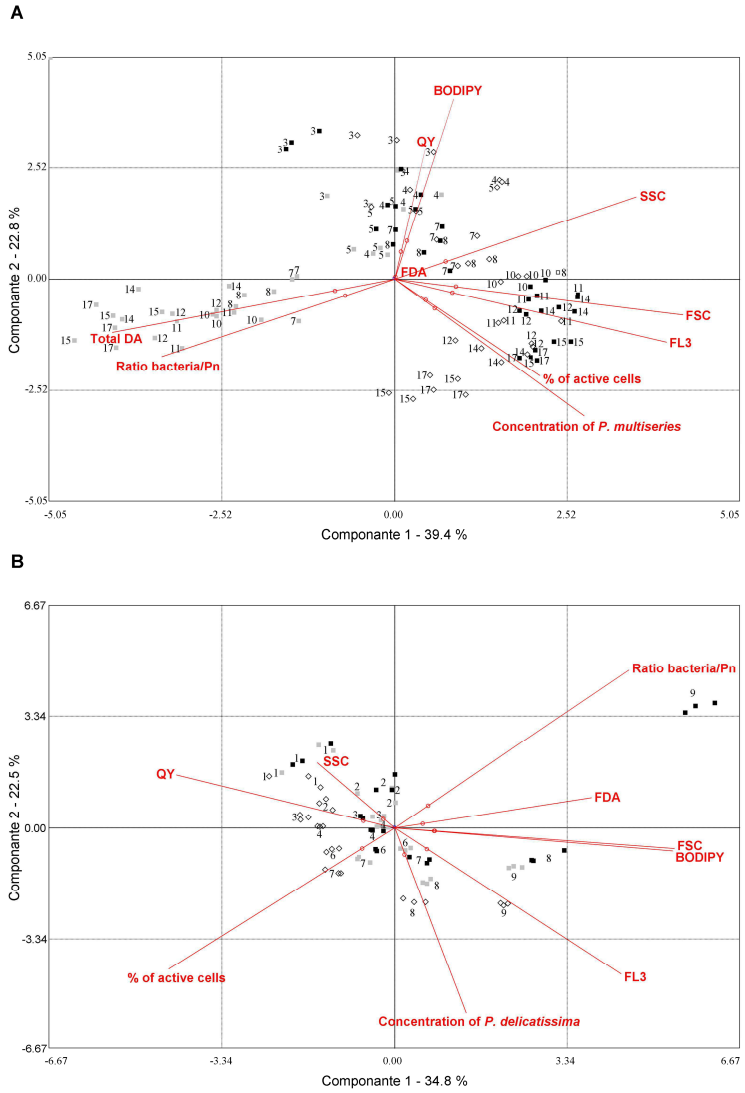

Figure 6: Principal component analysis on *P. multiseries* (A) and *P. delicatissima* (B) grown without bacteria (open diamonds), with bacterial community of *P. multiseries* (filled squares) or with bacterial community of *P. delicatissima* (grey squares).

Discussion

4.1. Effect of algae on bacteria

Antibiotic treatment killed almost all bacteria of *P. delicatissima* and even if the treated culture was not fully axenic, the bacterial community was reduced enough to consider the culture as "axenic" for the time of the experiment, compared to the non treated-culture (bacteria could not be counted until day 7). Unfortunately, cells of *P. multiseries* were much more sensitive to AB; AB concentration had thus to be reduced and more bacteria survived to the treatment. Even though, bacterial concentration was reduced by 10-fold on day 0, the effect of bacteria has to be considered. As M0 cultures were not axenic at the beginning, bacteria remained all along the experiment.

The absence of growth of *P. delicatissima* associated bacteria with *P. multiseries* cells may be due to the algae themselves, or to the remaining *P. multiseries* bacteria. When grown with *P. delicatissima*, bacteria of *P. delicatissima* grew faster than bacteria of *P. multiseries*. Bacteria of *P. multiseries* grew however faster when associated to *P. delicatissima* cells than *P. multiseries* cells. The culture media was exactly the same for each condition, and *P. delicatissima* was also the algae that grew the fastest, probably acquiring nutrients from the water faster. Competition can therefore not explain the differences between the two *Pseudo-nitzschia* species. Bacteria had two distinct growth phases, with a faster growth during the second phase. The second phase of growth started during the mid-exponential phase of the algae, regardless of the algae and the bacteria, which can be related to a higher release of exudates in the media as algal cells were more numerous. Indeed, the excretion of organic carbon by natural algal community accounted on average for 22 % of the bacterial carbon demand on a river system (Descy et al., 2002) and up to 79 % of the algal exudates can be incorporated by bacteria (Petit et al., 1999). Kirkwood et al. (2006) found that exudates of different cyanobacteria enhanced growth of bacteria with a wide range of response, even between species of the same genus. Exudates of algae could actually explain the differences of growth rate of bacteria between the two cultures; *P. delicatissima* probably released more exudates than *P. multiseries*. None of our algal species seemed to have bactericidal effects, as percentage of dead bacteria was the same for each *Pseudo-nitzschia* species and bacterial community. Nevertheless, algae had effects on bacteria, as bacterial communities exhibited different morphological parameters depending on the algae they were grown with. Indeed, FSC and SSC of bacteria decreased when grown with *P. delicatissima* while FSC and SSC of bacteria increased when grown with *P. multiseries*. These modifications can be explained by aggregation or disaggregation of bacteria, morphological modifications (e.g. lengthening of bacteria) or selection of different species.

4.2. Effect of a reduced bacterial concentration

Both *Pseudo-nitzschia* species grew for at least 2 years in culture before these experiments and were thus adapted to "their" bacterial community. The AB treatment had effects on the algal cells, as observed by the higher sensitivity of *P. multiseries* to AB (data not shown). Cultures were inoculated with bacteria two days after the end of the AB treatment. As *P. delicatissima* grew with a growth rate of $\mu = 1.0\ d^{-1}$, which represents ~3 generations a day, effect of AB on algal physiology was highly reduced before the beginning of the experiment,

except on day 1, where the high percentage of dead cells probably represented cells permeabilized by AB and not dead cells. Cultures of *P. multiseries* grew slower but were exposed to lower doses of AB. Due to the age of the isolate, around 20 % of cells were dead in untreated cultures of *P. multiseries* (data not shown). The high percentage of cells stained at the beginning of the experiment was also due to AB permeabilisation of cell membranes. Nevertheless, for each species, all the cultures experienced the same AB treatment, thus comparison can be performed between cultures with or without bacteria.

When bacteria were removed from the cultures (almost fully or only partially for *P. delicatissima* and *P. multiseries* respectively), growth rate of *Pseudo-nitzschia* was enhanced and cultures reached a higher maximum cell concentration. Bates et al. (1995) found that xenic cultures reached a higher cell concentration than axenic cultures, whereas Osada and Stewart (1997) axenic cultures reached a higher cell concentration than xenic culture. But, they both found that xenic and axenic cultures grew with a similar growth rate. On the opposite, Douglas et al. (1993) found that an axenic culture had a higher growth rate than control cultures. In this study, the percentage of dead cells was slightly lower in axenic cultures, but the difference was not significant, suggesting that bacteria do not have any algicidal effect. The increase of maximum cell concentration could be due to the absence of competition (both for nutrients and space) or to the fact that bacteria, if not algicidal, were at least able to limit algal growth.

If cell growth was enhanced, DA production of "axenic" *P. multiseries* was decreased to almost no production (< 0.02 pg cell^{-1}, 10-fold less than control cultures). Cultures of *P. delicatissima* did not produce DA, with or without bacteria. This decrease of DA production in axenic cultures has already been shown in previous studies (Bates et al., 1995; Douglas et al., 1993; Osada and Stewart, 1997). As previously demonstrated by Bates et al. (2004), DA is not produced by bacteria but by *P. multiseries* cells themselves. Bacteria removal reduced DA production by *P. multiseries* and increased its growth rate and cell concentration while *P. delicatissima* only reached a higher maximum concentration. The increase of *P. multiseries* growth rate without bacteria could also be explained by the fact that cells that did not use their energy for DA production and thus re-allocated it to growth processes. Nevertheless, the removal of all the bacteria was not necessary to limit DA production, as "axenic" cultures of *P. multiseries* had bacteria (10 % of xenic cultures on day 0 and 100 % on day 13). This could be explained by the fact that only some bacteria can induce DA production and that these

bacteria have been killed by AB. Indeed, Osada and Stewart (1997) showed that only bacteria producing gluconolactone/gluconic acid, a chelatant of metals, were able to induce DA production. AB treatment may indeed have killed all the bacteria producing gluconolactone/gluconic acid and associated to *P. multiseries.*

Both FSC and SSC of algal cells were reduced in "axenic" cultures, regardless of the species. This decrease of morphological parameters may be linked to a decrease of the number of epibiontic bacteria after AB treatment, which did not come back, as bacteria were not added, or to a modification of the cytoplasmic organisation, which was shown to be related to variation of FSC and SSC (Lelong et al., 2011). Indeed, FSC and SSC are measurements of laser deviation and presence of epibiontic bacteria can probably modify them. The percentage of active cells was not modified without bacteria but cells of *P. multiseries* had a lower esterase activity without bacteria. As the competition with bacteria was lower and DA production reduced, cells may not have needed a high esterase activity to sustain their higher growth rate. Indeed, esterase activity has been shown to increase when *P. multiseries* cells started to produce DA (Lelong et al., 2011), probably because cells enhance the production of enzymes needed to produce DA and esterases are also enhanced, in a direct or indirect pathway. Thus if cells have less DA, they also have a lower esterase activity. Moreover, cells of *P. delicatissima* did not produce any DA and their esterase activity was not modified. Cells of *P. multiseries* and *P. delicatissima* had also fewer lipids when bacteria were removed. As "axenic" cultures were growing faster, they used more energy and had fewer lipids to store. The difference could also be due to bacterial lipids. Indeed, lipids of epibiontic bacteria were probably stained and may have been included in algal lipid content. To our knowledge, no study has been published so far on lipid content of the marine bacterial community, thus without any values, it is difficult to know if bacterial lipids would be high enough to enhance BODIPY fluorescence or not. The chlorophyll content of cells and the efficiency of photosynthesis were not modified either. Cells were able to perform photosynthesis, acquire carbon and probably acquire energy by respiration within the same proportion with or without bacteria. Photosynthetic efficiency of *P. delicatissima* was slightly reduced without bacteria, but not enough to affect the cells, as quantum yield remained above 0.5 during the entire exponential phase. With or without bacteria, cells did not exhibit much physiological modifications, meaning that even if cells grew better without bacteria, they were accustomed to "their" bacteria, with an adapted physiology.

4.3. Effect of exchanged bacterial community

Cultures of *P. multiseries* were unable to grow when cultivated with *P. delicatissima* bacteria and *P. delicatissima* cultivated with *P. multiseries* bacteria, even if they grew well, had no stationary phase and died right after exponential phase. Different species of bacteria induced different effects on algal cells, from enhancement to inhibition of algal growth (Grossart and Simon, 2007). Grossart and Simon (2007) found that the addition of natural bacterial community to axenic *Thalassiosira rotula* increased the growth but cells died faster than in control cultures. Bacteria of *P. delicatissima* inhibited the growth of *P. multiseries*, and caused the algal cells to die when the concentration of *P. delicatissima* bacteria increased. Moreover, cells of *P. multiseries* started to die. Cultures of *P. delicatissima* grown with *P. multiseries* bacteria had no stationary phase, a lower maximum concentration and an increased percentage of dead cells. Cells probably stopped growing as they became more sensitive to bacteria, which may indeed be directly responsible for algal death. The exchange of bacteria modified the growth and survival of the two *Pseudo-nitzschia* species, to a different extent. The two bacterial communities seem to have algicidal effects, which may also explain why cells without bacteria grew better and faster than cells grown with their bacteria. It also confirms that each species is "adapted" to its bacterial community. The physiology of these cells was thus followed, to assess whether these modifications could be related to the physiological status of cells and if the physiology of cells could be related to DA production.

The bacterial community did not modify the chlorophyll content or the photosynthetic efficiency of *P. delicatissima* cells, which can explain why cells were growing with the same growth rate regardless of the associated bacteria. When looking at their primary metabolism during exponential phase, esterase activity was not modified. The lipid content of *P. delicatissima* grown with alien bacteria was exactly the same than those of cells grown with *P. delicatissima* bacteria. As for the physiological parameters, the morphological parameters, linked, at least partially, to the cell content were not modified. These *P. delicatissima* cells, which were not able to produce any DA, probably found another mechanism to compete bacteria, which seems really efficient regardless of the associated bacterial community. When cells started to die, they exhibited an increased esterase activity, which may be a non specific mechanism induced to counteract mechanisms inducing cell death when cells are exposed to a stress. During several months after the isolation of the strain, cultures of *P. delicatissima* did not exhibit any stationary phase and died in a similar manner than *P. delicatissima* grown

with *P. multiseries* bacteria (Lelong A., unpublished data). A stationary phase appeared finally after around a year in our culture conditions. We can thus hypothesise that *P. delicatissima*, regardless of the associated bacterial community, needed several months to get adapted to this bacterial community and the appearance of a stationary phase proved that algal cells can adapt to and counteract algicidal affect of bacteria.

Bacterial community had much more effect on the physiology of *P. multiseries*. Indeed, *P. multiseries* cells grown with *P. delicatissima* bacteria had a reduced chlorophyll content and photosynthetic efficiency, as well as a reduced lipid content and an increased esterase activity at the beginning of the exponential phase. Cells of *P. multiseries* with *P. delicatissima* bacteria did not seem able to perform efficient photosynthesis, and thus acquired less carbon and probably less energy through respiration. This energy was allocated over the first days to primary metabolism and more precisely to esterase activity, and not growth, as well as to secondary metabolism with an increased DA production. Lelong et al. (2011) showed that the beginning of DA production by *P. multiseries* is associated to an increase of esterase activity. Lipids were not stored, even if the cells were stressed, as cells did not acquire a lot of energy and probably used everything for DA production, at the expense of growth. After the mid-exponential growth phase, all the energy was allocated to DA production. DA concentration was up to 17-fold higher than *P. multiseries* grown with their "own" bacterial community, and no storage of lipids nor esterase activity nor growth could be observed. Bates et al. (1995) found that DA production was enhanced by 115-fold following the addition of alien bacterial community, isolated from *Chaetoceros* sp. More recently, Kaczmarska et al. (2005) proposed that a specific bacterial community composition, or its density, may be important to explain the variable DA levels associated with some *P. multiseries* cultures. They hypothesized that some bacteria were antagonistic to the algae and induced an increase in DA production as a specific response to the presence of bacteria. Bacterial density was identical regardless of their origin, thus bacterial composition more likely triggered DA production. Wells et al. (2005) also suggested that algae produced DA for trace metal acquisition. But in the present study, *P. multiseries* did not produced DA to outcompete bacteria for trace metal acquisition, as algal cells did not grow and died. It can be due to algicidal effect of bacteria, or to the fact that all the energy they acquired (probably not a lot as chlorophyll content was reduced) was allocated to DA production. To explain this difference, we can hypothesise that cells of *P. multiseries* are not accustomed to bacteria of *P. delicatissima*, possibly because of

gluconolactone/gluconic acid production by bacteria but not only or necessarily (Osada and Stewart, 1997; Stewart et al., 1997).

Cells of *P. multiseries* grown with "their" bacteria produced some DA, at decreasing concentration per cell as cells were growing. DA production decreased with reduced bacterial load and was enhanced by the addition of alien bacterial community. Thus, bacteria (alien or not) induced a stress; stress that induced DA production with a re-allocation of energy and a decrease of growth. Cells of *P. delicatissima* did not produce any DA and their physiology was not modified upon addition of alien bacteria. They were likely less stressed and/or developed mechanisms/strategies to compete the alien bacteria during exponential phase. However, these mechanisms probably weakened them and led them to death right after exponential phase. All the experiments run to set off DA production by *P. delicatissima* failed (Fe and Cu limitations, Lelong et al., in prep; Cu toxicity, Lelong et al. 2012a). There are two main hypotheses to explain this difference between toxic and non-toxic species: (i) bacteria are not the first trigger of DA and DA production is indirectly enhanced by the stress resulting of bacterial growth and (ii) bacteria are one of the first DA trigger but DA production is a burden for cells resulting in slower growth in culture conditions. Indeed, toxic strains/species often grew more slowly than non-toxic strain/species, in the same conditions (Fehling et al., 2005; Thessen et al., 2009).

For *P. multiseries*, alien bacteria, although not growing, resulted in a very strong stress, stopping growth and triggering high production of DA (or triggering DA, which stopped growth). Toxic and non-toxic *Pseudo-nitzschia* species possibly possess different mechanisms to outcompete bacteria or at least tolerate the presence of bacteria in their media. In the first group (toxic species), it involves DA production but probably not only and in the second it involves other unknown defences mechanisms, likely more efficient as non-toxic strains/species most often grow faster than toxic ones when cultivated in the same conditions (or with alien bacterial community). In toxic species/strains, DA production is often associated to stressful conditions (limitation in micro or macro-nutrients). We can hypothesize that stressful conditions, resulting in limited/reduced growth, may favour bacteria development and thus trigger DA production.

Table 1: Physiological parameters of *P. multiseries* grown without bacteria (M0), with its bacteria (MM) or with bacteria of *P. delicatissima* (MD). FSC and SSC are morphological parameters measured by flow cytometry and expressed as arbitrary units. Chlorophyll autofluorescence is the FL3, expressed in arbitrary units and measured by flow cytometry. Quantum Yield has been measured by Pulse Amplitude Modulated fluorometry. Esterase activity has been measured by uptake of FDA and lipid content by BODIPY staining, both expressed in arbitrary units.

	day	FSC		SSC		Chlorophyll autofluorescence		Quantum Yield		FDA uptake		BODIPY fluorescence	
		mean	± SE	mean	± SE	mean	± SE	mean	± SE	mean	± SE	mean	± SE
M0	3	282.8	9.5	105.7	1.7	437.9	86.9	0.38	0.01	130.0	7.8	1466.4	65.6
	4	283.4	12.5	106.8	6.7	503.8	38.8	0.38	0.05	157.3	37.3	1516.1	123.7
	5	273.1	9.3	100.0	10.7	503.9	55.8	0.36	0.03	76.8	45.5	1530.4	83.2
	7	277.4	1.4	100.1	2.3	647.1	94.5	0.39	0.04	122.5	8.3	1099.5	123.6
	8	292.1	6.4	100.1	3.7	786.8	116.3	0.36	0.03	116.3	3.6	1121.6	63.7
	10	290.0	0.8	99.3	0.1	821.1	66.0	0.41	0.05	121.6	1.6	1013.9	41.5
	11	291.4	2.7	97.4	2.8	832.7	67.5	0.35	0.04	112.4	5.1	907.0	39.5
	12	290.5	5.5	95.8	3.5	793.3	101.1	0.33	0.05	111.1	1.5	826.9	33.0
	14	290.1	3.0	92.7	1.6	812.2	24.9	0.31	0.05	126.6	9.2	913.2	81.8
	15	289.1	2.6	83.5	6.1	511.6	187.8	0.22	0.01	106.2	7.4	822.7	16.3
	17	285.0	2.8	90.3	2.8	622.2	21.3	0.23	0.01	131.9	15.6	785.7	28.7
MM	3	265.9	3.4	97.1	6.1	364.5	36.2	0.40	0.02	149.3	13.2	1443.7	42.7
	4	280.8	2.7	96.2	3.6	472.6	33.8	0.39	0.00	132.7	11.4	1572.0	212.6
	5	269.4	2.4	99.7	1.7	474.9	55.1	0.39	0.02	114.2	8.5	1336.8	171.1
	7	281.9	6.8	98.1	5.3	643.3	35.3	0.42	0.02	139.5	6.4	1099.4	255.4
	8	280.3	4.5	97.6	3.0	641.5	42.2	0.37	0.02	129.4	7.9	1193.9	67.1
	10	299.0	3.3	105.1	2.6	921.4	6.2	0.35	0.01	148.9	9.0	1092.6	30.8
	11	302.3	4.6	105.7	4.0	906.9	62.5	0.35	0.01	137.3	8.7	1019.8	11.4
	12	300.5	1.7	105.5	2.9	853.7	44.8	0.30	0.01	128.7	9.7	999.3	11.6
	14	305.6	3.6	102.5	3.2	978.6	47.4	0.31	0.02	161.6	7.6	1240.1	20.7
	15	301.1	1.8	104.8	4.0	890.2	17.9	0.25	0.01	140.7	11.5	984.0	37.2
	17	307.4	3.5	105.0	3.1	842.3	41.9	0.21	0.01	174.0	13.8	989.5	3.7

	day	FSC		SSC		Chlorophyll autofluorescence		Quantum Yield		FDA uptake		BODIPY fluorescence	
		mean	± SE	mean	± SE	mean	± SE	mean	± SE	mean	± SE	mean	± SE
MD	3	270.8	5.7	99.3	6.4	549.4	35.5	0.34	0.02	138.7	20.8	1303.8	48.4
	4	269.1	8.8	98.3	1.1	631.2	84.8	0.29	0.02	167.2	27.7	1493.2	293.8
	5	261.3	1.4	91.5	1.8	689.1	68.9	0.29	0.00	187.1	15.3	1353.7	23.5
	7	252.9	4.0	87.6	2.4	569.5	49.3	0.22	0.07	198.5	21.5	1147.7	38.0
	8	254.0	3.8	87.3	2.7	510.7	47.9	0.25	0.02	159.9	7.4	1050.7	17.1
	10	256.1	7.4	88.7	3.5	466.1	29.0	0.24	0.02	145.2	15.1	961.0	19.2
	11	259.6	6.6	87.2	4.8	430.4	29.0	0.25	0.04	148.5	9.0	878.9	45.9
	12	255.4	4.4	87.0	2.9	414.2	26.3	0.26	0.02	141.9	9.9	912.5	141.7
	14	257.5	11.3	86.8	6.3	382.4	54.1	0.35	0.04	128.5	15.0	982.0	70.3
	15	256.1	11.1	83.1	4.7	346.0	82.9	0.38	0.07	114.1	7.7	794.7	30.1
	17	261.1	6.8	89.0	2.1	378.0	88.2	0.35	0.01	111.9	15.6	730.0	43.5

Table 2: Physiological parameters of *P. delicatissima* grown without bacteria (D0), with its bacteria (DD) or with bacteria of *P. multiseries* (DM). FSC and SSC are morphological parameters measured by flow cytometry and expressed as arbitrary units. Chlorophyll autofluorescence is the FL3, expressed in arbitrary units and measured by flow cytometry. Quantum Yield has been measured by Pulse Amplitude Modulated fluorometry. Esterase activity has been measured by uptake of FDA and lipid content by BODIPY staining, both expressed in arbitrary units.

	day	FSC		SSC		Chlorophyll autofluorescence		Quantum Yield		FDA uptake		BODIPY fluorescence	
		mean	± SE	mean	± SE	mean	± SE	mean	± SE	mean	± SE	m ean	± SE
D0	1	68.0	0.6	15.7	0.4	218.1	3.5	0.40	0.08	33.6	3.0	108.5	20.9
	2	75.2	0.7	15.3	0.4	252.8	4.9	0.56	0.01	46.5	0.4	124.9	13.6
	3	75.6	0.3	14.2	0.5	252.4	2.0	0.58	0.01	33.9	0.2	116.3	3.7
	4	76.9	0.3	13.1	0.2	256.9	3.8	0.59	0.00	34.9	1.8	124.9	4.3
	6	68.1	0.6	12.9	0.0	260.4	2.4	0.55	0.00	40.1	4.4	150.3	11.6
	7	67.0	0.2	13.5	0.4	253.7	1.2	0.40	0.01	47.4	0.2	95.5	13.2
	8	80.9	0.3	14.0	0.1	323.7	2.1	0.30	0.02	33.4	1.6	118.9	17.3
	9	88.0	0.2	15.1	0.3	339.2	4.5	0.26	0.01	34.2	1.6	207.7	10.6
DD	1	68.7	2.0	16.0	1.0	207.8	7.1	0.44	0.04	37.9	2.4	102.7	5.3
	2	81.8	1.0	16.3	0.4	270.7	5.8	0.55	0.02	51.1	4.6	138.4	27.3
	3	85.5	0.8	15.9	1.0	289.3	3.1	0.59	0.01	40.9	1.0	151.9	7.4
	4	86.8	0.5	14.5	0.1	283.2	3.5	0.62	0.01	37.8	4.5	161.8	4.6
	6	79.8	0.5	14.2	0.2	289.5	7.1	0.59	0.01	33.1	0.8	227.6	10.7
	7	73.3	0.4	13.8	0.3	258.4	2.8	0.48	0.02	38.2	1.7	158.6	8.3
	8	83.0	0.4	14.1	0.1	302.5	2.4	0.35	0.01	38.9	1.4	143.7	1.6
	9	88.8	0.4	15.0	0.2	309.9	3.3	0.30	0.01	45.4	1.0	199.8	5.6
DM	1	69.0	0.9	15.8	0.5	209.8	17.3	0.47	0.05	35.1	2.3	105.6	4.2
	2	83.1	0.3	16.8	0.8	275.6	10.3	0.56	0.01	52.0	3.4	128.8	23.9
	3	82.5	0.2	15.8	0.3	289.9	2.6	0.58	0.01	38.8	2.0	135.8	4.4
	4	84.4	0.3	14.4	0.1	293.9	3.0	0.63	0.00	40.5	0.8	152.0	15.3
	6	71.3	0.7	13.1	0.2	276.1	3.8	0.58	0.01	45.6	5.4	184.2	9.6
	7	77.3	0.4	14.7	0.3	299.6	7.4	0.44	0.02	60.2	2.0	148.8	5.5
	8	92.4	0.1	14.9	0.1	356.8	3.7	0.34	0.01	73.0	15.7	171.8	3.5
	9	92.2	0.4	12.7	0.2	275.3	1.5	0.30	0.00	59.5	9.5	242.9	4.7

Table 3: Morphological related parameters of bacterial community in *P. multiseries* cultures grown without bacteria (M0), with its bacteria (MM) or with bacteria of *P. delicatissima* (MD) and in *P. delicatissima* grown without bacteria (D0), with its bacteria (DD) or with bacteria of *P. multiseries* (DM).

	day	FSC bactéries		SSC bactéries	
		mean	± SE	mean	± SE
M0	3	91.2	2.3	2.9	0.0
	4	106.0	4.4	4.3	0.2
	5	74.9	2.7	4.5	0.1
	7	123.1	3.8	4.6	0.2
	8	260.7	7.8	4.7	0.1
	10	272.7	4.4	4.8	0.2
	11	212.8	9.2	4.0	0.0
	12	337.2	18.0	4.1	0.1
	14	198.1	0.9	3.8	0.1
	15	198.1	0.9	3.8	0.1
	17	369.4	18.7	4.2	0.4
MM	3	110.7	1.4	4.1	0.0
	4	128.8	1.1	4.1	0.1
	5	113.6	1.1	4.1	0.0
	7	173.1	5.5	4.6	0.0
	8	263.9	17.2	5.3	0.1
	10	292.4	43.4	5.5	0.2
	11	191.6	15.0	4.9	0.2
	12	262.9	21.3	5.4	0.3
	14	193.5	12.7	4.9	0.1
	15	193.5	12.7	4.9	0.1
	17	311.3	19.7	5.2	0.2
MD	3	91.7	3.2	7.9	0.5
	4	92.6	4.3	8.7	0.3
	5	79.5	5.1	7.9	0.1
	7	118.7	7.2	7.8	0.1
	8	180.9	9.4	7.3	0.0
	10	190.4	1.8	7.3	0.2
	11	145.0	7.3	5.5	0.1
	12	191.7	24.7	6.9	1.1
	14	193.1	7.3	4.6	0.1
	15	193.1	7.3	4.6	0.1
	17	329.0	10.3	5.4	0.8
D0	6	94.9	43.4	7.4	1.8
	7	423.0	138.7	12.1	1.1
	8	506.5	70.6	14.6	2.6
	9	446.1	135.9	16.2	3.4
DD	1	529.1	13.9	26.9	0.5
	2	316.2	8.8	19.4	0.3
	3	214.9	0.7	18.0	0.1
	4	193.0	2.3	16.0	0.1
	6	176.0	2.4	17.4	0.1
	7	177.1	2.2	17.6	0.2
	8	172.9	0.8	16.2	0.1
	9	136.6	3.1	13.2	0.1

	day	FSC bactéries		SSC bactéries	
		mean	± SE	mean	± SE
DM	1	306.0	1.3	16.5	0.2
	2	131.4	1.8	11.2	0.1
	3	95.0	2.2	10.8	0.1
	4	92.4	0.9	9.6	0.0
	6	118.3	1.6	9.3	0.0
	7	114.4	2.2	8.5	0.1
	8	111.5	1.1	8.2	0.0
	9	116.1	1.7	7.5	0.1

Acknowledgements

This work was supported by the French ministry of research (MENRT grant) to AL, Region Bretagne grant, SAD Volet 1, PSEUDOPE to HH and CNRS EC2CO Microbien to PS.

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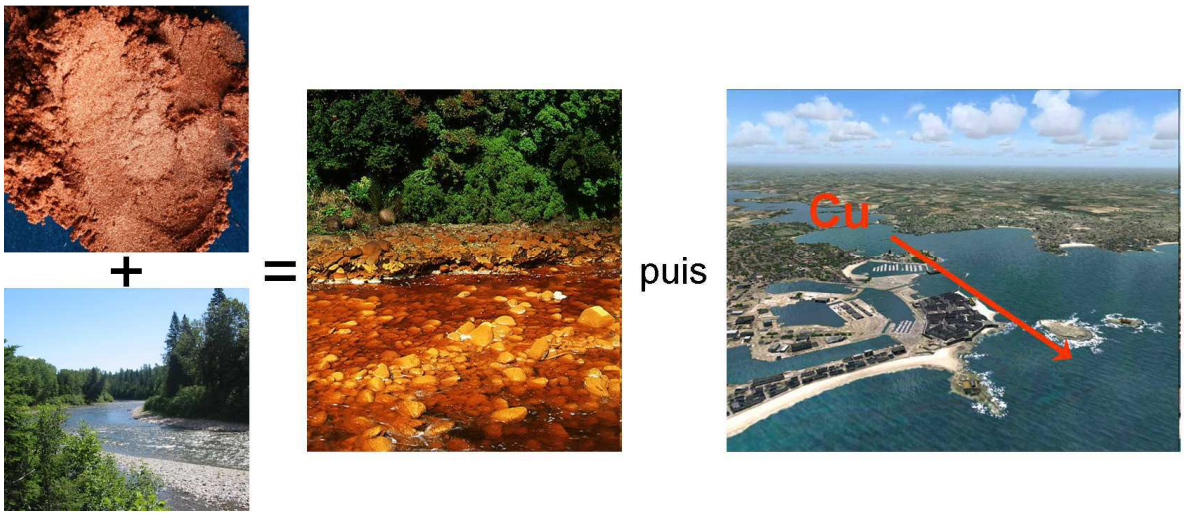
4.3. Conclusion

Le fait qu'une souche soit non-toxique n'est pas dû à la présence ou la nature des bactéries environnantes, tout au moins pour les souches et les communautés bactériennes que nous avons étudiées. En effet, dans notre étude, l'ajout de bactéries associées à une souche toxique de *P. multiseries* n'a pas pu rendre toxique une souche initialement non-toxique de *P. delicatissima*. A l'inverse les bactéries associées à une souche non-toxique peuvent augmenter la toxicité de la souche toxique. Par contre la présence de bactéries est bien indispensable à la production d'acide domoïque. Quelle que soit l'espèce, l'absence (ou la faible présence) de bactéries favorise la croissance phytoplanctonique et le métabolisme est modifié en conséquence (moins de cellules mortes, avec une plus faible activité des estérases et un moindre stockage de lipides). Le changement de communauté bactérienne affecte les deux espèces, mais de façon plus drastique *P. multiseries*. Il est cependant difficile de savoir si l'arrêt de la croissance des microalgues est dû à la production d'acide domoïque ou si la production d'acide domoïque est la conséquence de cette faible croissance. Il sera également important dans le futur de chercher à comprendre si la production d'acide domoïque est une cause directe ou indirecte de la présence de bactéries, à savoir donc si les bactéries induisent "volontairement" cette production ou si la seule compétition pour les nutriments est suffisante. Pour trouver la réponse à cette question, il s'avère très important de toujours prendre en compte, si ce n'est la nature des bactéries, au moins leur nombre, car cette étude montre qu'elles peuvent modifier la physiologie des cellules et la production d'acide domoïque.

La compétition avec les bactéries est bien un facteur qui augmente la production d'acide domoïque. S'il s'agit de compétition pour l'acquisition de micronutriments, comment des cellules de *Pseudo-nitzschia* modifient leur métabolisme pour répondre à des conditions de disponibilité en métaux traces différentes ? La production d'acide domoïque protège-elle les cellules de la toxicité du cuivre ?

EFFETS DE DOSES TOXIQUES DE CUIVRE SUR *PSEUDO-NITZSCHIA* SPP.

5



Article :

Lelong A., Jolley D., Hégaret H., Soudant P. 2012. The effects of copper toxicity on *Pseudo-nitzschia* spp. physiology and domoic acid production. *Aquatic toxicology* 118-119: 37-47.

5.1. Préambule

En zones côtières habitées et industrialisées, le ravinement des eaux de pluie et le rejet des eaux usées peuvent entraîner des quantités importantes de métaux, et notamment de cuivre, vers l'océan (Stephenson and Leonard, 1994). Les concentrations de cuivre peuvent ainsi atteindre 1-2 µg l-1 dans les eaux côtières (Morel and Price, 2003), ou encore entre 0.2 et 50 µg l^{-1} en Méditerranée (Santos-Echeandia et al., 2008) contre moins de 0.25 µg l^{-1} en océan ouvert (Apte and Day, 1998) et entraînent des effets délétères sur les espèces de microalgues côtières. Le seuil de tolérance au cuivre, qui provoque une inhibition de 50 % de la croissance, peut varier d'un facteur 1000 selon les espèces phytoplanctoniques (0.6 µg Cu l^{-1} pour *Minutocellus polymorphus*, Levy et al., 2007, et 635 µg Cu l^{-1} pour *Parachlorella kessleri*, Nugroho and Frank, 2011). Quand ce seuil est dépassé, les espèces ne poussent plus puis meurent. L'acide domoïque est connu pour être un chélatant non seulement du fer mais également du cuivre (Rue and Bruland, 2001). L'une des hypothèses de la communauté scientifique est que le cuivre ainsi chélaté n'est plus biodisponible, ce qui diminue sa toxicité (Maldonado et al., 2002). Le premier objectif de ce chapitre est donc de vérifier si l'acide domoïque est produit pour protéger les cellules contre une dose toxique de cuivre. Pour cela des doses toxiques de cuivre ont été ajoutées au milieu de culture d'une espèce toxique (*P. multiseries*) et d'une espèce non-toxique (*P. delicatissima*). Pour vérifier le rôle de l'acide domoïque en tant qu'agent protecteur contre le cuivre, lors d'une deuxième expérience, de l'acide domoïque a été rajouté à des cultures de *P. delicatissima* en même temps que des doses croissantes de cuivre.

5.2. Article 5 – *Impact of copper exposure on* Pseudo-nitzschia *spp. physiology and domoic acid production*

Aquatic Toxicology 118–119 (2012) 37–47

Contents lists available at SciVerse ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aquatox

Impact of copper exposure on *Pseudo-nitzschia* spp. physiology and domoic acid production

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ARTICLE INFO

Article history:
Received 14 October 2011
Received in revised form 15 March 2012
Accepted 17 March 2012

Keywords:
Pseudo-nitzschia
Diatom
Copper toxicity
Flow cytometry
Physiology
Domoic acid

ABSTRACT

Microalgae have differing sensitivities to copper toxicity. Some species within the genus *Pseudo-nitzschia* produce domoic acid (DA), a phycotoxin that has been hypothesised to chelate Cu and ameliorate Cu toxicity to the cells. To better characterise the effect of Cu on *Pseudo-nitzschia*, a toxic strain of *P. multiseries* and a non-toxic strain of *P. delicatissima* were exposed to Cu(II) for 96 h (50 μg l^{-1} for *P. delicatissima* and 50, 100 and 150 μg l^{-1} for *P. multiseries*). Physiological measurements were performed daily on *Pseudo-nitzschia* cells using fluorescent probes and flow cytometry to determine the cell density, lipid concentration, chlorophyll autofluorescence, esterase activity, percentage of dead algal cells, and number of living and dead bacteria. Photosynthetic efficiency and O_2 consumption and production of cells were also measured using pulse amplitude modulated fluorometry and SDR Oxygen Sensor dish. The DA content was measured using ELISA kits. After 48 h of Cu exposure, *P. delicatissima* mortality increased dramatically whereas *P. multiseries* survival was unchanged (in comparison to control cells). Cellular esterase activity, chlorophyll autofluorescence, and lipid content significantly increased upon Cu exposure in comparison to control cells (24 h for *P. delicatissima*, up to 96 h for *P. multiseries*). Bacterial concentrations in *P. multiseries* decreased significantly when exposed to Cu, whereas bacterial concentrations were similar between control and exposed populations of *P. delicatissima*. DA concentrations in *P. multiseries* were not modified by Cu exposure. Addition of DA to non-toxic *P. delicatissima* did not enhance cells survival; hence, extracellular DA does not protect *Pseudo-nitzschia* spp. against copper toxicity. Results suggested that cells of *P. delicatissima* are much more sensitive to Cu than *P. multiseries*. This difference is probably not related to the ability of *P. multiseries* to produce DA but could be explained by species differences in copper sensitivity, or a difference of bacterial community between the algal species.

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1. Introduction

In coastal waters, copper concentrations fluctuate due to both natural and anthropogenic activities, such as volcanoes, industrial discharges (Paul and Pillai, 1983), mining (Castilla, 1996; Stauber et al., 2000), algaecides (McKnight et al., 1983) and antifouling paints (Hall and Anderson, 1999). Copper is an essential trace metal for microalgae, as a component of key proteins such as Cu-superoxide dismutase, cytochrome c oxidase and plastocyanin (Raven et al., 1999; Balamurugan and Schaffner, 2006; Peers and Price, 2006). However, at higher concentrations, copper becomes toxic inducing a range of metabolic changes in phytoplanktonic cultures, such as inhibition of cell division (Lage et al., 2001; Perales-Vela et al., 2007), photosynthesis (Perales-Vela et al., 2007), respiration (Xia and Tian, 2009), pigments synthesis (Perales-Vela et al., 2007), cell mobility inhibition (Lage et al., 2001) and changes in membrane potential (Cid et al., 1996). Copper toxicity is likely to occur through mechanisms such as inhibition of enzymes and oxidative damage within the cell (Knauert and Knauer, 2008). Copper sensitivity in microalgae varies vastly between species, with dissolved copper inhibiting population growth by 50% (IC-50) at concentrations < 1 μg $Cu\,l^{-1}$ in *Minutocellus polymorphus* (Levy et al., 2007), and 630 μg $Cu\,l^{-1}$ in *Parachlorella kessleri* (Nugroho and Frank, 2011).

The effects of copper toxicity in microalgae have been widely studied, mainly to identify potential bioindicators of contamination (e.g. Ahmed and Hader, 2010). These studies typically focus measurements on population growth and cell death, sometimes observing physiological changes such as photosynthetic rates or ATP production (Cid et al., 1995). Only a few studies have investigated the effects of metal exposure on *Pseudo-nitzschia* species, most of which were centred on domoic acid (DA) production

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doi:10.1016/j.aquatox.2012.03.010

(Ladizinsky, 2003; Maldonado et al., 2002). DA is an amnesic toxin, which chelates both copper and iron (Rue and Bruland, 2001), and is produced by some species of *Pseudo-nitzschia* (see a review in Lelong et al., 2012). Maldonado et al. (2002) hypothesised that DA is produced in response to copper exposure to chelate copper, reducing its toxicity to cells by decreasing its bioavailability. *Pseudo-nitzschia multiseries* and *P. australis* have been shown to release approximately 20-fold more DA when exposed to toxic concentrations of copper (Ladizinsky, 2003; Maldonado et al., 2002). The effects of copper exposure on toxic and non-toxic *Pseudo-nitzschia* species have not been reported in the literature. A comprehensive understanding of factors governing DA production in *Pseudo-nitzschia* spp. is of global interest to commercial fisheries, as bivalves, molluscs and fishes contaminated by DA pose a threat to human health (Bejarano et al., 2008). So far, the factors triggering DA production in algae remains unclear, but as Cu concentrations can become elevated in coastal waters, particularly following pulse wastewater discharges from industry (Paul and Pillai, 1983), it is important to know if DA is produced by cells in response to copper exposure.

This study investigated the effects of copper toxicity on the physiology of two species of *Pseudo-nitzschia*: a non-toxic (non-DA producing) *P. delicatissima* and a toxic (DA producing) *P. multiseries*. The first step was to examine the impacts of copper on different physiological parameters simultaneously, to allow comparison between the two species. All physiological measurements were performed during acute (24 h) and chronic (24–96 h) copper stress. Flow cytometry was used to measure the concentration and percentage of dead *Pseudo-nitzschia* and associated free-living bacteria, and the lipid content and enzymatic activity of algal cells. A pulse-amplitude modulated (PAM) fluorometer and an oxygen sensor dish (SDR) were used to measure photosynthesis efficiency and quantify O_2 consumption and release during cell respiration and photosynthesis. For each experiment, DA was also quantified to estimate its potential as a protective agent against copper. To fully evaluate the protective role of DA in copper toxicity, DA was added to *P. delicatissima* cultures to allow a comparison with the DA producing *P. multiseries* cultures.

2. Material and methods

2.1. Culture conditions

The two species of *Pseudo-nitzschia* used in this study were *P. multiseries* (strain CLNN-16, isolated from the Bay of Fundy, Canada) and *P. delicatissima* (strain Pd08RB, isolated from the Rade de Brest, France). Both strains were 2.5 years old at the time of the experiment. They were grown in sterilised f/2 medium (Guillard and Hargraves, 1993) at 17.2 °C (±0.5 °C) and 155 ± 10 μmol photons m^{-2} s^{-1} (with a light: dark photoperiod of 12:12 h). Cultures were xenic and grown without antibiotics.

2.2. Bioassays

Experiments were always performed on cultures in exponential growth phase (3–5 days old). Before each experiment, cultures were homogenised by gentle manual stirring. Aliquots of the cultures were centrifuged 3 times (5 min, 16 °C, 780 g) and the remaining pellet was rinsed with sterile seawater (>0.22 μm filtered, Sartorius bottle-top filter). After the last rinse, the pellet was resuspended in sterile seawater. Almost all the cells in the cultures were present as single cells, with <5% forming two cell chains. Therefore, for flow cytometry analyses, they were all considered as single cells.

Cells were inoculated into flasks (TPP cell culture flasks 75 cm^2, Dutscher, Brumath, France) at a cell concentration of ~5000 cells ml^{-1}. Nutrient enriched sterile seawater (containing 26 μM $NaNO_3$, 13 μM NaH_2PO_4 and 10.6 μM Na_2SiO_3) was added to each inoculate. A stock solution of 50 mg Cu l^{-1} was prepared from $CuSO_4 \cdot 5H_2O$ in 0.1% of HCl. Copper exposure concentrations were selected based on Maldonado et al. (2002). Treatments were prepared in triplicate at 0 (controls), 50, 100 or 150 μg l^{-1} for both *P. multiseries* and *P. delicatissima*, however *P. delicatissima* proved to be more sensitive to copper and only 50 μg l^{-1} exposures are reported here. The flasks were incubated for 4 days (96 h) under the above conditions. Flasks were rotated within the cabinet and shaken twice daily by hand to ensure sufficient gas exchange. The pH remained at 8.0 ± 0.2 throughout the test (recorded initially and at test completion).

2.3. Copper analyses

Copper measurements were conducted using an octopol collision cell-inductively coupled plasma-mass spectrometer (OCR-ICP-MS, Agilent 7500cs) utilising both standard and collision/reaction gas modes where applicable. Calibration standards were prepared in 0.32 M HNO_3 (Choice Analytical, Suprapur, 69%) using a multi-element standard (IV-ICP-MS-71D, Inorganic Ventures, USA). Blanks, duplicates and spiked recoveries were performed on at least 10% of all samples. Method blanks were below the 0.89 μg l^{-1} limits of reporting, duplicates were within 12% of each other and spike-recoveries were 92–107%. On day 4, samples were filtered (0.45 μm, Minisart syringe filter, Sartorius), acidified to 0.32 M HNO_3 and copper was measured.

2.4. Specific growth rate, morphological and physiological measurements

Specific growth rate (μ, d^{-1}) was determined by linear regression of the natural log of cell concentration (cell ml^{-1}) over time (days). A flow cytometer FACScalibur (BD Biosciences, San Jose, CA, USA) with an argon blue laser (488 nm) was used. Flow cytometer flow-rates were calculated daily by analysing samples for 45 s, as per Marie et al. (1999). Morphological information of cell complexity was determined using forward scatter (FSC) and side scatter (SSC) as *Pseudo-nitzschia* is a pennate diatom, not a spherical cell. FL3 fluorescence (red fluorescence at 670 nm) was also determined as an indicator of cell chlorophyll content, as Galbraith et al. (1988) showed that autofluorescence of cells is linearly linked to chlorophyll content.

Cell density, morphological and physiological measurements of *Pseudo-nitzschia* spp., quantification of free-living bacteria associated to *Pseudo-nitzschia* populations and percentage of dead bacteria in the treatments were assessed using fluorescent stains and flow cytometry according to Lelong et al. (2011). A range of commercial stains from Invitrogen (Molecular Probes, Invitrogen, Eugene, Oregon, USA) were used to assess physiological changes associated with copper exposure in *P. delicatissima* and *P. multiseries*. Physiological changes included mortality (30 min incubation in 0.1 μM of SYTOX Green), intracellular lipid content (30 min incubation in 10 μM of BODIPY 493/503), and esterase activity (6 min incubation in 3 μM of fluorescein di-acetate (FDA)). A 300 μM working solution of FDA was freshly prepared before each experiment. Cells were considered dead when they were permeable to SYTOX Green (Fig. 1A and B). Esterase activity is a measure of metabolic activity (Jochem, 1999) and cells with no FDA staining were considered as inactive cells (as opposed to stained and active cells, Fig. 1C and D). The concentration of free-living bacteria (both living and dead) were measured after 15 min incubation with SYBR Green I (a 1/10,000 dilution of the commercial solution), and

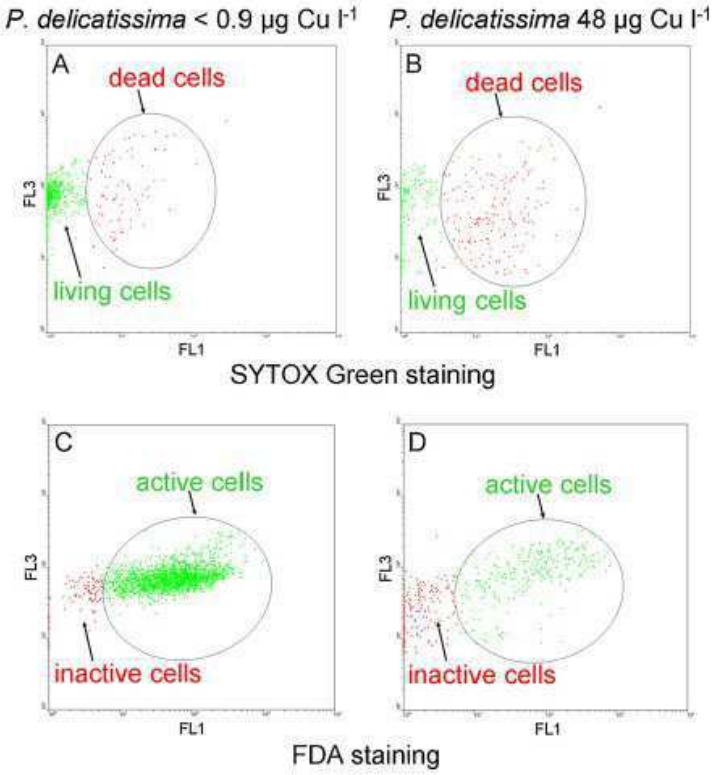

Fig. 1. Dotplot of *P. delicatissima* cultures obtained after SYTOX Green staining (A, B) or FDA staining (C, D) for control cultures (A, C) or cultures treated with 48 µg l^{-1} of copper (B, D). Each point represents a cell. Circled populations are dead cells (A, B) or active cells (C, D) while non-circled populations are living cells (A, B) or inactive cells (C, D).

propidium iodide (PI, 10 µg ml^{-1}, Sigma, St. Louis, MO, USA). Bacteria stained with PI were considered dead and percentage of dead bacteria was calculated using PI stained bacteria versus total number of bacteria.

Quantum Yield (QY) is a measure of the efficiency of the photosynthesis, where F_0 and F_m are the minimum and maximum fluorescence of cells at 455 nm, respectively. The measurement of QY was performed on cells after 20 min of dark adaptation at 17 °C. QY ($(F_m - F_0)/F_m$) of *P. multiseries* was measured using the PAM AquaPen-C AP-C 100 fluorometer (Photo Systems Instruments, Czech Republic).

Consumption or production of oxygen in each treatment was measured with an Oxygen SensorDish/*Reader*/® (SDR, Presens, Regensburg, Germany). Two aliquots of each flask were transferred to washed vials, one kept in the dark (respiration/consumption of O_2 only) and one in the light (photosynthesis and respiration) for 2 h. Gross production of O_2 attributable to photosynthesis in each treatment was determined by difference (light minus dark O_2). Consumption or production of O_2 was divided by the active cell concentration to obtain a mean rate per active cell per hour.

2.5. Domoic acid quantification

DA was quantified using ASP ELISA kit (Biosense Laboratories, Bergen, Norway) following manufacturer's instructions. Each replicate from each treatment was analysed in duplicate for quality control purposes. An aliquot was taken from each flask, filtered (0.22 µm, Minisart syringe filter, Sartorius), and the filtrate analysed for dissolved DA (DA_d). A second aliquot was sonicated on ice for 2 min to disrupt cell membranes and liberate DA from the cells, this fraction measured the total DA (DA_t). Cellular DA (DA_c) was obtained by difference, $DA_c = DA_t - DA_d$. Measurements of DA at 24 h were not presented, as 24 h was insufficient time for cells to produce detectable levels of DA in the test solutions (limit of detection was 300 ng DA l^{-1}).

2.6. Assessment of the potential protective effect of DA

To assess potential protective effects of DA against copper toxicity, *P. delicatissima* cells were exposed to different concentrations of copper and DA (domoic acid standard for ASP ELISA kit, Biosense Laboratories, Bergen, Norway). The DA concentrations used in this experiment corresponded to 0.5-, 1- and 2-fold the concentration of dissolved DA produced by *P. multiseries* during the earlier exposures to copper. Thus *P. delicatissima* cells were exposed to 0, 25, 50 and 100 µg l^{-1} of copper, and for each copper concentration, DA was added at 0, 0.25, 0.50 or 1.00 pg $cell^{-1}$ which corresponded to 0.75, 1.5 or 3.0 ng DA ml^{-1}, respectively. DA was added when cells were inoculated into the nutrient enriched sterile seawater at 3000 cells ml^{-1}. All treatments were performed in triplicate.

2.7. Statistics

Effects of copper on *P. multiseries* were tested using one way ANOVAs with the software StatGraphics Plus (Manugistics, Inc, Rockville, MD, USA). Variance homogeneity was tested, and Tukey's

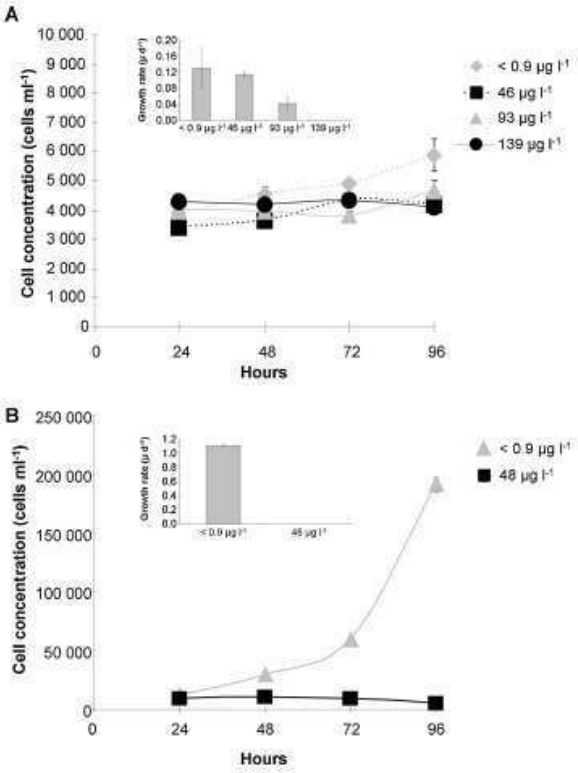

Fig. 2. Growth curves of *P. multiseries* (A) and *P. delicatissima* (B) during 96 h exposure to different doses of copper. 96-h growth rates (μ, d^{-1}) are indicated within each curve. For *P. multiseries* exposed to 46 $\mu g\,l^{-1}$ of copper, growth rate was measured during the first 72 h of growth. Mean ± SE, $n = 3$.

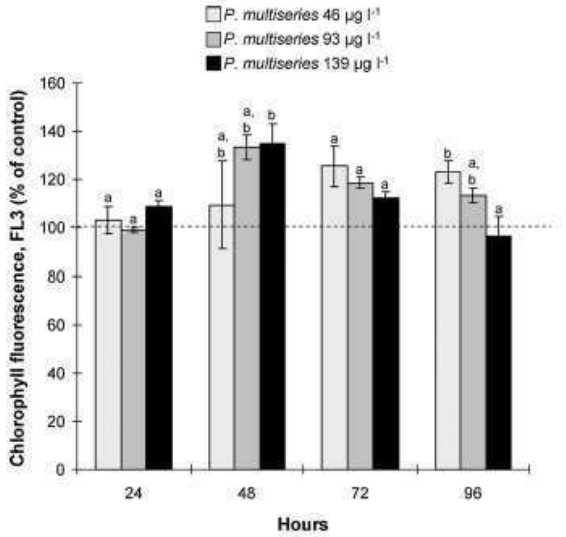

Fig. 3. Chlorophyll autofluorescence (FL3) of *P. multiseries* during 96 h exposure to different doses of copper. Results are expressed as percentage of control. Letters indicate values significantly different from each other (control is considered as group a), with each time of exposure treated separately. Mean ± SE, $n = 3$.

test was applied for parametric data. When the variance was not homogenous, the non-parametric test of Kruskal–Wallis was used. Significant differences in physiological parameters of *P. delicatissima* were assessed using the Student's *t*-test. Multifactor ANOVAs were performed on parametric data to assess the effect of time (hours) and copper treatment on population growth, and the effect of copper and DA concentrations on *P. delicatissima* (during assessment of protective effect of DA). Statistics were always performed on gross data even when presented as a percentage of the control, and each day separately. For all statistical results, a probability of $p < 0.05$ was considered significant.

3. Results

3.1. Growth rate and percentages of dead and inactive cells

Copper exposure inhibited the population growth of both *P. multiseries* and *P. delicatissima*. The 96-h dissolved copper concentrations for *P. multiseries* were <0.89 (control), 45.9 ± 0.6, 93.1 ± 0.7 and 138.6 ± 1.3 $\mu g\,Cu\,l^{-1}$ (mean ± SE, $n = 3$). Cells of *P. multiseries* exposed to 0 and 46 $\mu g\,Cu\,l^{-1}$ had similar 72-h growth rates, with growth rate dropping off for the Cu-exposed cells by 96 h (Fig. 2A). Cultures exposed to 93 $\mu g\,Cu\,l^{-1}$ had a significantly lower 96-h growth rate while the 139 $\mu g\,Cu\,l^{-1}$ populations did not grow past 96 h (Fig. 2A).

The 96-h dissolved copper concentrations for *P. delicatissima* were <0.89 (control) and 48.4 ± 0.3 $\mu g\,Cu\,l^{-1}$ (mean ± SE, $n = 3$). Control cells of *P. delicatissima* grew at 0.88 d^{-1}, whereas treatments exposed to 48 $\mu g\,Cu\,l^{-1}$ did not grow (Fig. 2B), with population density progressively decreasing over 4 days (from 8.8×10^3 cells ml^{-1} at 24 h to 4.9×10^3 cells ml^{-1} at 96 h).

The percentage of *P. multiseries* cells that were metabolically active (as determined by FDA staining, showing esterase activity), or dead (membranes permeable to SYTOX Green staining), did not vary significantly between controls and copper-exposed cells throughout the experiment (Table 1, $p > 0.05$). The proportion of dead *P. delicatissima* cells in copper exposed treatments increased significantly ($p < 0.05$) from 15 to 66% at 24 h and 96 h, respectively, whereas the proportion of dead cells in controls remained constant over the 96 h with between 5 and 12% dead cells (Table 2). The percentage of inactive *P. delicatissima* cells remained constant at approximately 2% in control cells whereas it increased from 2% to 30% in Cu-exposed cells at 24 h and 96 h, respectively (Table 2). For both *P. delicatissima* and *P. multiseries*, the percentage of dead cells was 3- to 4-fold higher than the percentage of esterase inactive cells after 96 h of exposure to copper.

3.2. Photosynthetic parameters

For *P. multiseries* cells, there was a 24 h lag phase during which the chlorophyll autofluorescence (as FL3 fluorescence) of the control and Cu-treated cells was not significantly different. However, after 24 h copper treated cells typically presented higher chlorophyll autofluorescence than control cells, with the 139 $\mu g\,Cu\,l^{-1}$ population returning to control levels of chlorophyll by 96 h (Fig. 3). The chlorophyll autofluorescence of *P. delicatissima* was significantly higher in Cu-exposed cells for the first 72 h ($p < 0.01$, Table 2), but decreased over 96 h in both control and Cu-exposed treatments.

The photosynthetic efficiency (as QY) of *P. multiseries* was consistently higher in control cells than copper treated cells, with significant differences emerging after the 24 h lag phase (Fig. 4). The concentration-dependent response to copper also became more apparent over time, with the 93 and 139 $\mu g\,Cu\,l^{-1}$ populations expressing much poorer (30–50% less) photosynthetic efficiency than controls and 46 $\mu g\,Cu\,l^{-1}$ populations after 72 and 96 h exposures. In the same way, the photosynthesis efficiency (as QY) of *P.*

Table 1
The effects of a 96 h exposure of *P. multiseries* to different doses of copper on the percentage of dead (determined after SYTOX Green staining) and inactive cells (determined after FDA staining) and on the morphological parameters of living cells (FSC and SSC).

	24 h			48 h			72 h			96 h		
	Mean	±SE		Mean	±SE		Mean	±SE		Mean	±SE	
% dead cells												
<0.9 µg l^{-1}	20.9	1.2	b	18.4	2.6	a	21.2	1.7	a	14.1	1.7	a
46 µg l^{-1}	19.1	1.2	ab	ND	ND	a	16.3	1.2	a	11.0	1.5	a
93 µg l^{-1}	14.9	1.2	a	22.8	1.9	a	18.2	2.0	a	13.5	2.3	a
139 µg l^{-1}	19.1	0.1	ab	22.2	2.0	a	18.9	3.1	a	15.8	2.0	a
% inactives cells												
<0.9 µg l^{-1}	17.5	1.8	a	9.8	1.0	a	6.3	0.8	a	3.6	1.1	a
46 µg l^{-1}	13.4	1.6	ab	4.7	0.5	b	2.3	1.3	b	2.7	0.8	a
93 µg l^{-1}	11.4	2.0	b	3.3	1.4	b	5.7	0.3	a	3.9	0.8	a
139 µg l^{-1}	14.6	0.4	ab	6.1	1.7	ab	4.6	0.6	ab	5.1	0.5	a
FSC												
<0.9 µg l^{-1}	287.4	2.2	a	263.1	1.9	b	311.9	3.5	b	300.8	1.7	a
46 µg l^{-1}	278.6	3.6	ab	245.1	3.5	a	299.8	3.9	ab	301.7	2.2	a
93 µg l^{-1}	276.0	2.6	b	297.8	5.5	c	293.5	3.8	a	292.3	2.1	a
139 µg l^{-1}	276.0	2.9	b	295.8	3.9	c	298.2	8.6	ab	296.8	5.9	a
SSC												
<0.9 µg l^{-1}	89.1	0.4	a	74.4	1.4	b	110.7	1.5	a	100.2	3.0	a
46 µg l^{-1}	86.3	1.1	a	63.3	2.7	a	101.3	5.5	ab	103.6	2.0	a
93 µg l^{-1}	87.4	1.2	a	93.7	1.7	c	95.9	3.4	b	99.6	1.5	a
139 µg l^{-1}	86.5	1.3	a	94.5	3.2	c	97.7	0.9	b	101.5	0.2	a

a, b and c correspond to statistical groups, with $p>0.05$ within groups, N.D.: no data.

delicatissima cells was consistently higher in control cells than copper exposed cells (Table 2), with significant differences ($p < 0.05$) emerging after 24 h (QY of 0.61–0.62 in control cells and 0.39–0.45 in copper-exposed cells from 48 to 96 h).

In *P. multiseries*, Cu-exposure decreased both O_2 consumption (due to respiration) and O_2 production (due to photosynthesis) rates, with the 93 and 139 µg Cu l^{-1} populations having consistently lower rates than controls (Fig. 5). The 46 µg Cu l^{-1} population of *P. multiseries* had decreased rates at 48 and 72 h exposures, however these were similar to control cell rates. The O_2 production of Cu-exposed *P. delicatissima* was not detectable, while O_2 consumption increased after 48 and 72 h of exposure to copper (with no detectable concentrations at 96 h, attributable to low cell densities, Table 2).

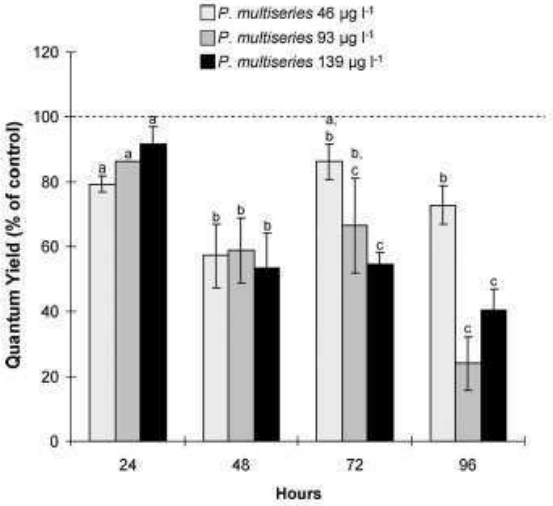

Fig. 4. Efficiency of photosynthesis (Quantum Yield, QY) of *P. multiseries* during 96 h exposure to different doses of copper. Results are expressed as percentage of control. Letters indicate values significantly different from each other (control is considered as group a), with each time of exposure treated separately. Mean ± SE, $n = 3$.

3.3. Physiological parameters

In *P. multiseries*, esterase activity was consistently higher in copper exposed cells in comparison to controls (Fig. 6). The

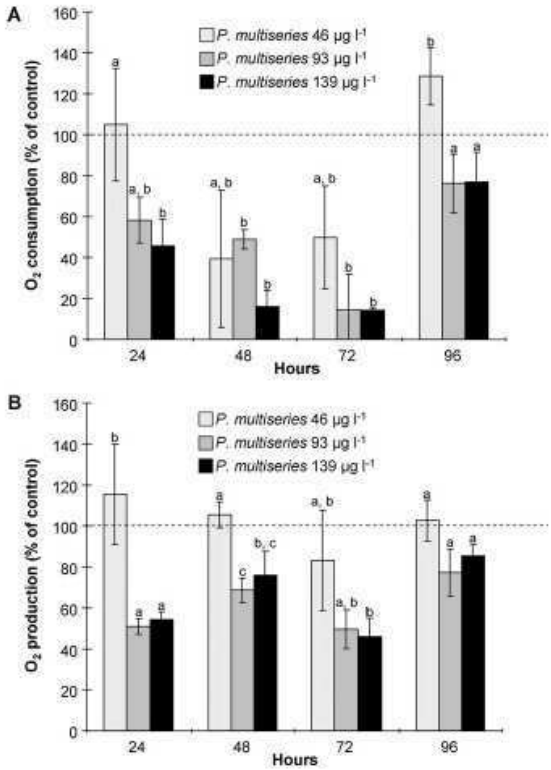

Fig. 5. O_2 consumption during respiration (A) and O_2 production during photosynthesis (B) of *P. multiseries* during 96 h exposure to different doses of copper. Results are expressed as percentage of control. Letters indicate values significantly different from each other (control is considered as group a), with each time of exposure

Table 2
The effects of a 96 h exposure of *P. delicatissima* to different doses of copper on the percentage of dead (determined after SYTOX Green staining) and inactive cells (determined after FDA staining), the cells autofluorescence (FL3), the Quantum Yield (QY), the O_2 consumption, the esterase activity (after FDA staining), the lipid content (after BODIPY staining) and on the morphological parameters of living cells (FSC and SSC).

	24 h					48 h					72 h					96 h				
	<0.9 µg l^{-1}		48 µg l^{-1}			<0.9 µg l^{-1}		48 µg l^{-1}			<0.9 µg l^{-1}		48 µg l^{-1}			<0.9 µg l^{-1}		48 µg l^{-1}		
	Mean	±SE	Mean	±SE		Mean	±SE	Mean	±SE		Mean	±SE	Mean	±SE		Mean	±SE	Mean	±SE	
% dead cells	12.2	1.0	14.8	0.6	NS	12.4	1.3	40.0	0.4	**	6.9	0.2	48.3	8.8	**	4.5	0.2	66.3	2.3	**
% inactive cells	2.2	0.5	2.3	0.4	NS	2.4	0.3	9.1	0.5	**	1.1	0.1	22.1	1.1	**	1.4	0.3	30.4	0.2	**
FL3 (AU)	18.3.3	6.2	230.0	0.1	**	144.2	2.5	192.5	6.1	**	107.3	1.9	128.7	3.4	**	69.4	0.7	72.0	4.1	NS
FL3 (% of the control)			125.7	4.1	**			133.7	5.8	**			120.2	5.2	**			103.8	6.9	NS
QY	0.46	0.02	0.43	0.02	NS	0.62	0.02	0.45	0.03	**	0.61	0.01	0.39	0.03	**	0.62	0.01	0.42	0.04	**
QY (% of the control)			94.0	7.8	NS			71.9	3.1	**			63.2	4.5	**			67.3	6.6	**
O_2 consumption (10^{-6} µmol h^{-1} cell^{-1})	0.396	0.048	0.504	0.117	NS	0.220	0.029	0.737	0.101	**	0.200	0.033	0.797	0.088	**					
Esterase activity (AU)	73.6	3.2	125.0	6.3	**	51.2	2.5	50.5	3.4	NS	126.9	8.5	55.7	3.8	**	59.9	3.0	20.7	1.0	**
Esterase activity (% of the control)			170.7	12.8	**			99.0	7.3	NS			44.6	1.2	**			34.6	1.5	**
Lipid content (AU)	194.6	2.2	286.5	3.2	**	195.5	1.9	332.2	4.2	**	148.1	8.4	306.6	1.48	**	105.9	4.1	222.4	3.7	**
Lipid content (% of the control)			147.3	2.7	**			169.95	1.88	**			212.7	0.5	**			210.4	7.0	**
FSC	86.0	1.9	110.7	0.4	**	55.5	0.67	75.6	1.6	**	58.6	0.7	73.5	0.5	**	57.3	0.5	71.5	3.5	**
SSC	83.7	1.7	89.6	6.4	*	13.3	0.1	14.8	0.3	**	12.5	0.1	12.6	0.3	NS	11.0	0.0	11.2	0.6	NS

* corresponds to statistical differences of $p < 0.05$ and ** of $p < 0.01$.

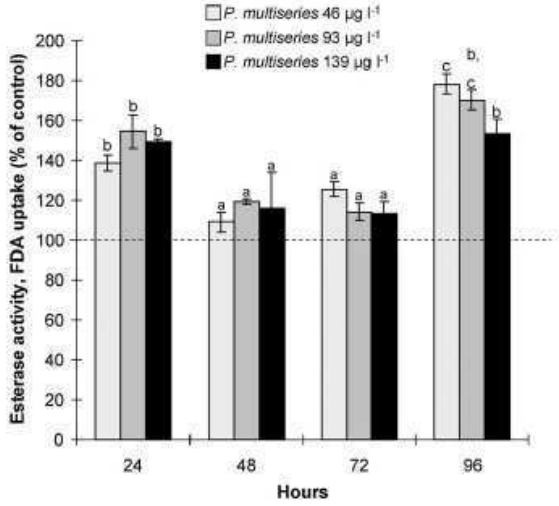

Fig. 6. Esterase activity (estimated from FDA uptake) of *P. multiseries* during 96 h exposure to different doses of copper. Results are expressed as percentage of control. Letters indicate values significantly different from each other (control is considered as group a), with each time of exposure treated separately. Mean ± SE, $n = 3$.

dose-dependent response to copper of esterase activity became apparent over time, with the 93 and 139 µg Cu l^{-1} populations expressing less esterase activity than the 46 µg Cu l^{-1} populations after 72 and 96 h exposures. Copper induced a varied esterase activity in *P. delicatissima* over the 96 h (Table 2). Esterase activity was stimulated in copper exposed cells at 24 h (71% higher than controls), however, this activity rapidly decreased to control levels at 48 h, then to significantly less than controls by 72 and 96 h (45 and 35% of controls, respectively, $p < 0.01$).

The 24-h lag phase was again evident in the response of *P. multiseries* cellular storage lipids (BODIPY) to copper exposure. No differences were observed between controls and any of the copper exposed treatments at 24 h, however after this time copper exposed cells had a higher lipid content ($p < 0.05$, Fig. 7). The 93 and 139 µg Cu l^{-1} populations produced significantly more lipids per cell than the 46 µg Cu l^{-1} populations by 72 and 96 h. This trend was also observed in *P. delicatissima*, in which the lipid content of *P. delicatissima* was consistently higher in cells exposed to copper throughout the experiment ($p < 0.01$, Table 2), increasing to twice the lipid content of controls by 72 h.

3.4. Morphological parameters

Morphological parameters of *P. multiseries* (FSC and SSC) did not significantly change between treatments (Table 1). In contrast, *P. delicatissima* FSC was significantly higher in copper-exposed cells in comparison to controls (Table 2). *P. delicatissima* SSC was also significantly higher in copper-exposed cells for the first 48 h (Table 2, $p < 0.01$).

3.5. Bacteria

Free-living bacteria associated with *P. multiseries* maintained a growth rate of 0.44 ± 0.01 d^{-1} in the control treatments throughout the experiments, with few dead bacteria (<5%). Bacterial concentrations in copper-exposed treatments decreased between 24 and 72 h, with a dose-dependent effect and an increase in the percentage of dead bacteria ($p < 0.05$). After 72 h, bacterial concentrations in copper-exposed treatments started to increase. Growth

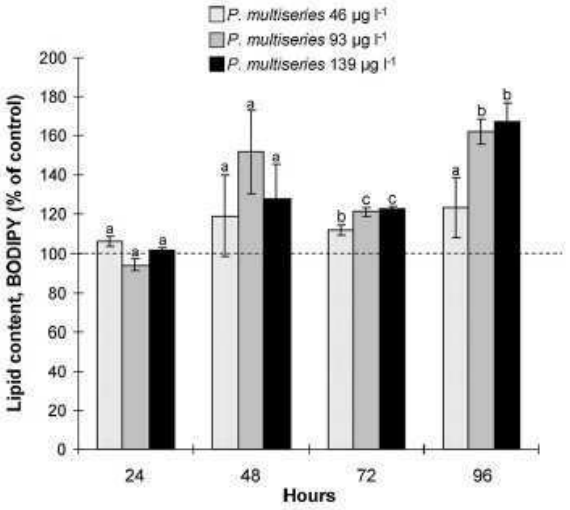

Fig. 7. Lipid content (estimated from the fluorescence of BODIPY, a lipid stain) of *P. multiseries* during 96 h exposure to different doses of copper. Results are expressed as percentage of control. Letters indicate values significantly different from each other (control is considered as group a), with each time of exposure treated separately. Mean ± SE, $n = 3$.

rates of free-living bacteria associated with *P. delicatissima* cells were not significantly modified by exposure to copper and was $1.00 \pm 0.03\ d^{-1}$ for control treatments and $0.91 \pm 0.03\ d^{-1}$ for copper exposed cells. There were no significant differences between the percentage of live bacteria in control and copper exposed treatments.

3.6. Domoic acid

Cellular and dissolved DA concentrations of *P. multiseries* were not significantly different between control and Cu-treated cells, irrespective of the time of exposure ($p > 0.05$, Fig. 8). Dissolved DA remained relatively constant at $0.50 \pm 0.07\ pg\ cell^{-1}$ from 48 h onwards, with no significant trends related to dose or exposure time. At 48 and 72 h, dissolved DA did not change with copper treatment (Fig. 8B). At 96 h, the amount of dissolved DA tended to be lower in copper-exposed cultures (in $pg\ ml^{-1}$) but there were also fewer cells producing it. Cells of *P. delicatissima* remained non-toxic with DA concentrations below the detection limit regardless of the treatment.

3.7. Assessment of the potential protective effect of DA

Increasing copper concentrations induced a decrease of both growth rate and percentage of living *P. delicatissima* cells, independent of DA concentration (Table 3). Extracellular DA had no significant effect on growth rate nor on the percentage of living *P. delicatissima* cells ($p > 0.05$). There was no interacting effect between copper and DA concentration detected in the multifactor ANOVA.

4. Discussion

Copper exposure had a significant effect on population growth, inhibiting the growth of both *P. multiseries* and *P. delicatissima*. Control cells of *P. delicatissima* grew at rates indicative of a healthy population. However, exposures as low as $48\ \mu g\ Cu\ l^{-1}$ prevented population growth, and increased the proportion of dead cells to

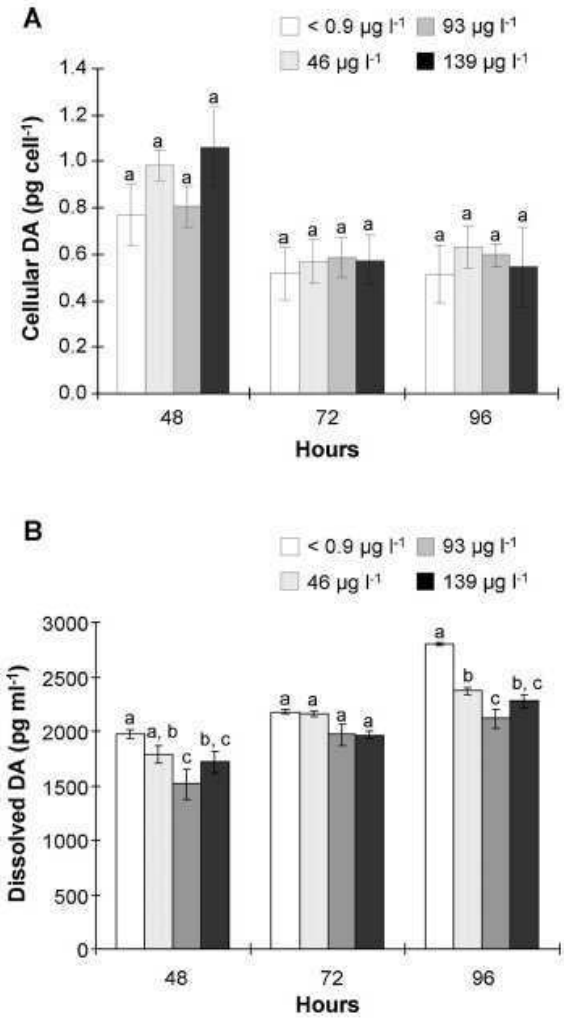

Fig. 8. Domoic acid (DA) content of *P. multiseries*, both inside cells (cellular DA, A) and outside cells (dissolved DA, B) during 96 h exposure to different doses of copper. Results at 24 h are not shown as cells just started to excrete DA after centrifugations made to prepare the experiment. Mean ± SE, $n = 3$.

66% by the end of the 96 h study. *P. multiseries* exposed to 0 and $46\ \mu g\ Cu\ l^{-1}$ had lower growth rates ($0.13\ d^{-1}$ and $0.12\ d^{-1}$, respectively) than those previously reported in the literature for other strains of this species (e.g. $0.8\ d^{-1}$, Bates et al., 2001; $0.92\ d^{-1}$, Kotaki et al., 1999; $0.24\ d^{-1}$, Lelong et al., 2011; $1.0\ d^{-1}$, Lundholm et al., 2004; $0.45–0.8\ d^{-1}$, Thessen et al., 2009). Growth rates of *P. multiseries* were consistent with cells grown in f/2 media before the experiment, indicating that control cells were healthy. It is possible that our lower growth rates could be explained by differences in the age of the isolates, which were not reported in the literature. The isolate used in this study was 2.5 years old, and the culture had maintained consistent growth rates over this time. Complete growth inhibition was observed in *P. multiseries* populations exposed to $\geq 139\ \mu g\ Cu\ l^{-1}$. Growth rates of *P. delicatissima* were much higher in the second experiment than the first one, because of the handling stress induced by centrifuging cells prior to inoculation in the first experiment.

Table 3
The effect of a 96 h of exposure to copper and domoic acid (DA) on the growth rate (μ, d^{-1}) of *P. delicatissima* during exposure to copper. Mean ± SE, $n = 3$.

DA (pg cell^{-1})	Cu concentration ($\mu g l^{-1}$)							
	0		25		50		100	
	Mean	±SE	Mean	±SE	Mean	±SE	Mean	±SE
0	1.29	0.01	0.78	0.07	0.29	0.02	0.07	0.02
0.25	1.57	0.02	0.81	0.02	0.24	0.02	0.06	0.05
0.5	1.55	0.04	0.77	0.11	0.22	0.08	0.12	0.05
1	1.58	0.01	0.83	0.18	0.53	0.00	0.06	0.01

Within a genus, species often have different sensitivities to copper, for example, the copper concentration inhibiting 50% of growth after 48 h was 5 μM for *Chlorella kessleri* (Kosakowska and Krol, 2009), 0.66 μM for *Chlorella vulgaris* (Hadjoudja et al., 2009), and 0.02 μM for the freshwater *Chlorella* sp. (Wilde et al., 2006). Within the *Pseudo-nitzschia* genus, only *P. multiseries* and *P. australis* have been studied for copper toxicity, with growth rates of *P. multiseries* and *P. australis* decreasing by 49% and 33%, respectively, following exposure to 1.8 μM of copper (~115 $\mu g l^{-1}$) (Maldonado et al., 2002). However, these concentrations were lethal for *P. delicatissima* in the present study, with 10–20% cells dead after 24 h, increasing to 66% after 96 h, as identified by the uptake of the cell permeant dye SYTOX Green. Our results are supported by Stauber and Florence (1985) who showed that copper increased cell permeability of microalgae, and Cid et al. (1996) who observed that cells of *Phaeodactylum tricornutum* exposed to 7.9–15.7 μM copper started to die after 48 h. It is also possible that copper compromises cell membrane integrity before apoptosis, which is why the percentage of dead cells was higher than the percentage of esterase inactive cells (Table 2). Hence some cells contained active esterases even though their membranes were permeable. Differences in copper sensitivity between our two strains can be related to species differences, strain difference or linked to DA production.

Both *P. multiseries* and *P. delicatissima* exhibited an increased chlorophyll autofluorescence when exposed to copper. A 24-h lag phase was observed for *P. multiseries* which was not evident for *P. delicatissima*. However, *P. multiseries* was more tolerant to copper, perhaps possessing cellular detoxification mechanisms which delayed the physiological effects of copper. This study is the first assessing the effects of copper exposure on chlorophyll autofluorescence in *Pseudo-nitzschia* species, thus it is only possible to compare our results to different genera. Franklin et al. (2001) found that chlorophyll autofluorescence can be both inhibited and stimulated by copper exposure, depending on the algal species and exposure concentration. *Microcystis aeruginosa* exposed to 0.5–2 μM of free copper for 48 h exhibited a decrease in chlorophyll autofluorescence (Hadjoudja et al., 2009), with an increase at 24 h for intermediate copper concentrations (1 and 1.3 μM). Cells with copper-induced growth inhibition had decreased concentrations of photosynthetic pigments in *Scenedesmus* sp. (i.e. chlorophyll *a* (chl *a*), chlorophyll b (chl *b*) and carotenoids, Tripathi and Gaur, 2006), increased carotenoid content in *C. vulgaris* (Mallick, 2004), and a decreased the ratio of Chl *a*/Chl *b* in *Scenedesmus vacuolatus* (Sabatini et al., 2009). Hence, the effect of copper on chlorophyll autofluorescence and pigments appears to be species-dependent, as well as being time- and concentration-dependent.

While the chlorophyll autofluorescence increased in both *Pseudo-nitzschia* species with copper exposure, the photosynthetic efficiency (as QY) for both species decreased, with a concentration-dependent response for *P. multiseries* at concentrations greater than 46 $\mu g l^{-1}$. QY is a proxy of the photosynthetic efficiency of cells at the photosystem II (PSII) level. It has been previously shown to decrease for cells exposed to copper, with a dose and time dependent effect (e.g. Yruela et al., 2000; Ahmed and Hader, 2010; Peña-Vázquez et al., 2010; Pérez et al., 2010), which is consistent with the results for *P. delicatissima* and *P. multiseries*. Xia and Tian (2009) found that photosynthesis of *Chlorella pyrenoidosa* was impacted at the PSII electron acceptor side, with a decrease in the O_2 production during photosynthesis after 12 h of exposure to 2–40 μM of copper. Rocchetta and Kupper (2009) found that the reaction of *Euglena gracilis* cells depended on copper concentration and time of exposure, without any clear pattern. Copper exposure decreased O_2 production in both *Pseudo-nitzschia* species, with production dropping below detectable levels for *P. delicatissima*. Respiration in *P. delicatissima* increased dramatically when exposed to copper, while it decreased in *P. multiseries*. Bacterial participation in this respiration should be considered in further experiments. Copper exposures that inhibited growth increased respiration rates between 12 h and several days after exposure for *E. gracilis* and *C. pyrenoidosa* (Rocchetta and Kupper, 2009; Xia and Tian, 2009).

The different responses to copper exposure were most likely related to the different sensitivities of the *Pseudo-nitzschia* species, as well as their associated bacterial communities. As *P. delicatissima* cells were dying, energy from their increased respiration may have been used to alleviate copper toxicity. This energy may have been used for primary metabolism; as suggested by esterases, which are part of this primary metabolism. Esterase activity was modified in *P. multiseries* and *P. delicatissima* cells exposed to copper. During the first 24 h of exposure to copper, both species exhibited an increase in their esterase activity, but after 48 h, this activity decreased in *P. delicatissima*, reaching 30% of controls. Esterase activity of *P. multiseries* remained higher in copper-exposed cells than in controls over the 96 h. Davis et al. (2006) showed that expression of Cu-induced genes (mainly unknown genes) in *Thalassiosira pseudonana* were enhanced during the first 24 h of exposure to copper and then decreased, as observed with esterase activity in our study. Hadjoudja et al. (2009) found that cells of *C. vulgaris* and *M. aeruginosa* exposed to copper enhanced their esterase activity after 5 h, which then decreased after 48 and 72 h. Lower copper concentrations induced higher esterase activity in our *P. multiseries* copper exposed treatments after 96 h, which was supported by a study of Hadjoudja et al. (2009), suggesting that esterase activity may not be related to cell death or growth. Increases in esterase activity relative to controls may be part of a non-specific pathway to detoxify or protect cells against a toxic chemical by enhancing different kinds of enzymes.

When microalgae are not able to divide, exhibit a reduced growth, or are undergoing a stress, they store their extra energy under lipid form (Giordano et al., 2001). Both *P. delicatissima* and *P. multiseries* exposed to copper exhibited an increase in lipid content, with a dose-dependent effect for *P. multiseries* after 72 h of exposure. If *P. delicatissima* cells increased their respiration under copper exposure, as found by Rocchetta and Kupper (2009) for *E. gracilis* and Xia and Tian (2009) for *C. pyrenoidosa*, and as growth rate was decreased, any extra energy produced could have been stored as lipid. An increase in cellular lipids was also observed in 9 species (of 11 tested) grown in NO_3^- deficient media (Palmucci et al., 2011).

Chlamydomonas reinhardtii and *Scenedesmus* sp. exposed to copper exhibited a decreased NO_3^- assimilation rate (Mosulen et al., 2003; Tripathi and Gaur, 2006). Hence there are possible links between copper exposure, NO_3^- assimilation and the production of cellular lipids. Further studies are needed to prove this link, and to determine the classes of lipids produced/modified by copper exposure.

The increase in lipid content would alter the morphological complexity of the cells (Lelong et al., 2011), thus partially explaining the increase in FSC and SSC morphological parameters of *P. delicatissima*. Changes in cell morphology (as increased FSC or SSC) have been previously observed in other algal species exposed to copper (Cid et al., 1996; Franklin et al., 2001; Araujo et al., 2010), and have been related to an increase in cell volume based on microscopic observations for *P. tricornutum* (Cid et al., 1996). An increase in FSC and SSC of *P. delicatissima* indicated that these cells may have been undergoing reorganisation and/or swelling of cell cytoplasm, perhaps due to cell permeabilisation. Further microscopic analyses are required to confirm this hypothesis.

P. delicatissima exist both as toxic (Rhodes et al., 1996; Lundholm et al., 1997) and non-toxic strains (Fehling et al., 2005; Thessen et al., 2005), as defined by DA production. The strain used in this study was non-toxic, and the exposure of *P. delicatissima* to elevated concentrations of copper did not induce any detectable DA production, hence copper exposure did not induce a non-toxic strain to become toxic. The strain CLNN-16 of *P. multiseries* was toxic; however, copper exposure did not modify DA production. Maldonado et al. (2002) found that *P. multiseries* exposed to 1.8 μM (115 $\mu g l^{-1}$) of copper increased DA production by 20-fold (from 5 to 105 amol DA $cell^{-1}$ h^{-1}) in comparison to control cells (~0.64 $\mu g l^{-1}$ of Cu). The Cu concentration used by Maldonado et al. (2002) was similar to that used in our study, however it did not induce DA production in our strain of *P. multiseries*. Even without copper, our strain (CLNN-16) was much more toxic (~1 pg $cell^{-1}$ of particulate DA) than that used by Maldonado et al. (2002) with ~20 fg $cell^{-1}$ of particulate DA, suggesting that CLNN-16 may not have had a physiological requirement to produce any additional DA to chelate copper. Indeed cultures of control and copper exposed cells in this study contained 2.8 $\mu g l^{-1}$ and 2.3 $\mu g l^{-1}$ of dissolved DA after 96 h, respectively, whereas in Maldonado et al. (2002) unexposed and Cu-exposed treatments contained ~0.2 $\mu g l^{-1}$ and 3.4–5.3 $\mu g l^{-1}$ of dissolved DA after 5 days, respectively. To identify if the amount of DA produced by *P. multiseries* was sufficient to protect cells against copper, DA was added with copper to the non-toxic *P. delicatissima*. No interaction could be observed between copper and DA, regardless of DA concentration (1/2 to 2-fold the concentration released per cell of *P. multiseries*). From this experiment, the presence of extracellular DA did not appear to explain the greater resistance of *P. multiseries* to copper exposure in comparison to *P. delicatissima*. The cells still died and growth was inhibited when exposed to copper. Nevertheless, DA is not only excreted, it can also stay inside the cells (~2/3 of the total DA in our experiment), hence it may be that intracellular DA is more efficient in protecting cells against Cu. The proportion of excreted DA did not change when *P. multiseries* were exposed to copper.

The difference between the *Pseudo-nitzschia* species' sensitivities to copper did not seem to be related to DA production. However, there were copper induced differences with the associated bacterial communities. Indeed, the bacterial community of *P. delicatissima* was resistant to copper, whereas the algae were highly sensitive. Conversely, the bacteria associated with *P. multiseries* were sensitive to copper and the algae resistant. Studies on marine bacteria are scarce and usually show a negative impact of copper on bacteria living both in sediments (Almeida et al., 2007) and seawater (Chandy, 1999). Marine bacteria have differing sensitivities to copper (Toes et al., 2008), with copper tolerant (Andreazza et al., 2010) and copper sensitive species (Ore et al., 2010). One hypothesis is that bacteria of *P. multiseries* produce chelating agents (e.g. siderophores) stronger than those of their algal counterparts, which render copper unavailable and less toxic for algae. In contrast, bacteria of *P. delicatissima* do not produce any relevant chelating agents, or if they are produced, they are less effective than those of the *P. multiseries* associated community – thus making copper available and toxic to the algae. Bates et al. (1995) and Kaczmarska et al. (2005) have reported that cells growing with bacteria induced DA production, depending on the bacterial community. Thus, the increased DA production observed by Maldonado et al. (2002) under copper exposure may have been induced by an increased bacterial concentration. However, they did not measure bacteria so this hypothesis cannot be verified. In our laboratories, stimulation of bacterial growth under copper exposure has been already observed in algae associated bacteria. Further, experimental differences could explain differences between our study and that of Maldonado et al. (2002), as procedures were not the same with the major difference being that Maldonado et al. (2002) used artificial seawater, allowing them to measure ionic activities, whereas we used natural seawater. Thus, even if the amount of copper added was identical, cells may have not experienced the same copper activity.

The two species exhibited different sensitivities to copper, thus the physiological response of *P. delicatissima* was characteristic of cells undergoing high stress, whereas that of *P. multiseries* indicated that it was undergoing much lower stress. Cells of *P. delicatissima* had two responses, one up to 24 h (acute stress) and one after 24 h (chronic stress). In the acute phase, cells did not die and exhibited an increase in their esterase activity and chlorophyll autofluorescence, with no modification of photosynthetic efficiency and consumption of O_2. In contrast, between 24 and 96 h, cells died, and their esterase activity, chlorophyll autofluorescence and efficiency of photosynthesis dramatically decreased. Nevertheless, consumption of O_2 increased. Lipid content and cell size increased under both acute and chronic copper stress. Cells of *P. multiseries* did not exhibit substantial differences between acute and chronic stress. Indeed, stress was lower than that experienced by *P. delicatissima*, as cells did not die. During acute stress, cell physiology of *P. multiseries* was less modified, with only a decrease in O_2 production and consumption and an increase of esterase activity. During chronic stress, two phases could be distinguished: between 48 and 72 h, chlorophyll autofluorescence and lipid content increased, photosynthetic efficiency, O_2 production and consumption decreased, and esterase activity was unchanged. At 96 h, chlorophyll autofluorescence and esterase activity were still higher than control but exhibited an inverse dose-related effect, with lower doses of copper inducing a higher response, while production of O_2 was identical to control cells and consumption of O_2 started to increase. It is possible that the detoxification mechanisms had been triggered to levels sufficient for the cells to manage that concentration of copper exposure, but this hypothesis needs to be further investigated. The bacterial response to copper exposure was opposite to that of the algal cells, with bacteria dying when *P. multiseries* grew and bacteria growing when *P. delicatissima* died. DA content (both cellular and dissolved) was not modified by copper exposure, strongly suggesting that DA had no protective effect against copper and was mainly beneficial to bacteria growth in the *P. delicatissima* treatments. Thus DA production did not explain the difference of sensitivity to copper between the toxic and non-toxic species. Bacterial community is the only difference observed in this study and it may explain copper sensitivity. Identification and subsequent investigations of the specific bacterial communities associated with each algal species could provide insight into this hypothesis. These results highlight the importance of considering bacteria when assessing copper toxicity. In addition to bacteria, there is likely to be a range of cellular mechanisms triggered by copper toxicity, which could explain differences

between species of the same genus, or even between strains of the same species.

Acknowledgements

This work was supported by the French Ministry of Research (MENRT grant), Region Bretagne grant, SAD Volet 1, PSEUDOPE to Hélène Hégaret and CNRS EC2CO Microbien to Philippe Soudant. We acknowledge the Australian Academy of Science for providing a 2010 International Science Linkages grant for Dianne Jolley.

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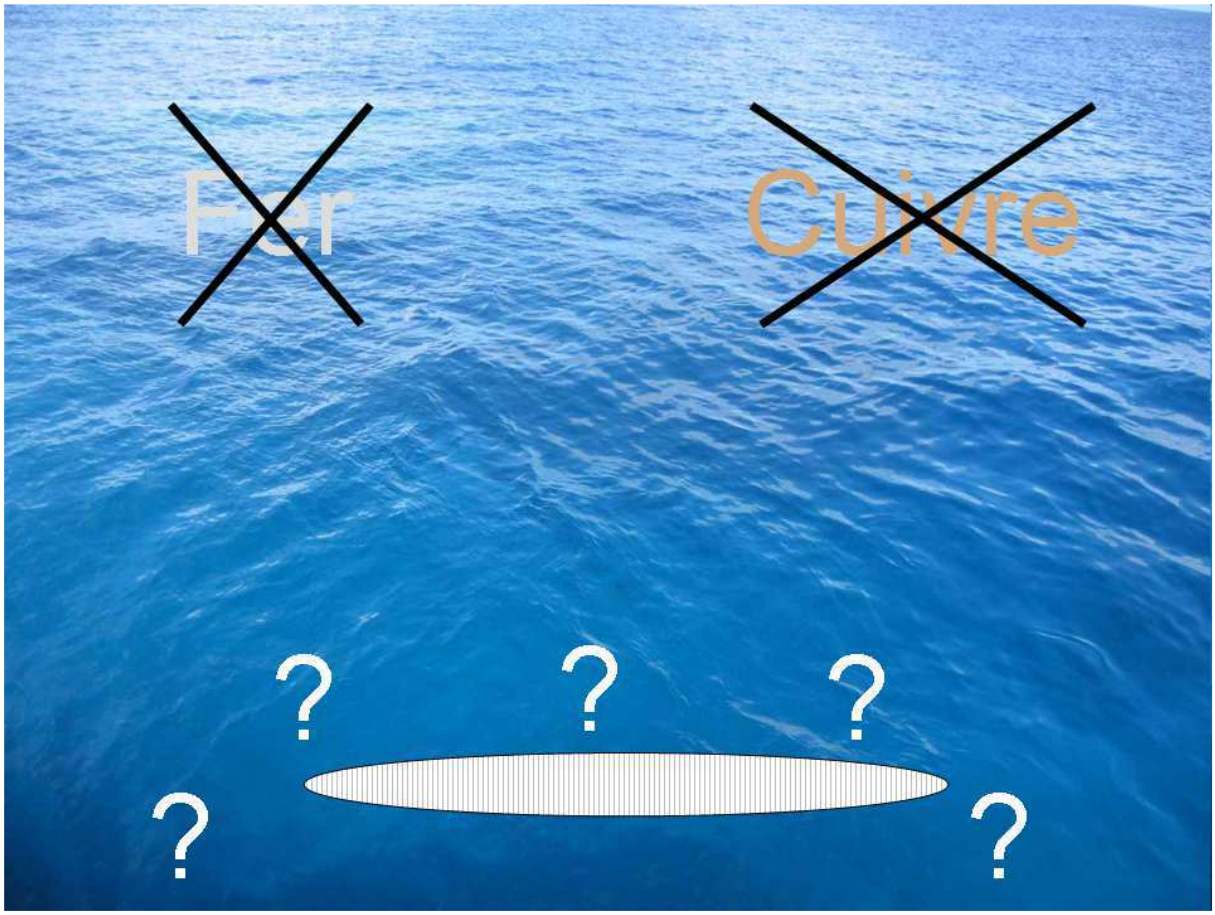
5.4. Conclusion

Cet article met en évidence l'impact de la toxicité du cuivre sur une espèce toxique et une espèce non toxique de *Pseudo-nitzschia*. L'espèce non-toxique *P. delicatissima* s'est avérée beaucoup plus sensible au cuivre que l'espèce toxique *P. multiseries*, une concentration de 50 µg l^{-1} de cuivre provoquant ainsi la mort de *P. delicatissima* et ne modifiant pas la croissance de *P. multiseries*. Les doses de cuivre utilisées pour *P. multiseries* (entre 50 et 150 µg Cu l^{-1}) étaient cependant suffisantes pour induire un stress, sa physiologie étant modifiée après l'ajout croissant de cuivre, avec notamment une augmentation du contenu lipidique, du métabolisme primaire et du contenu en chlorophylle *a* probablement induite pour compenser la diminution de l'efficacité de photosynthèse. Pour autant, la production d'acide domoïque par *P. multiseries* n'a pas été modifiée par l'ajout croissant de cuivre. L'ajout simultané d'acide domoïque et de cuivre à des cultures de *P. delicatissima* (non toxiques) a permis de montrer que l'acide domoïque ne protège pas les cellules de *Pseudo-nitzschia* contre la toxicité du cuivre. La différence de résistance de ces deux espèces au cuivre ne semble donc pas due à l'acide domoïque, mais à des mécanismes physiologiques différents, *P. multiseries* possédant peut être des mécanismes de détoxification plus efficaces que *P. delicatissima*. L'autre hypothèse soulevée par ce travail est que les bactéries pourraient être à l'origine de la différence de sensibilité des algues, les bactéries sensibles étant liées à des algues résistantes et inversement. Un résultat qui n'était pas attendu est l'effet favorable de l'acide domoïque sur la croissance des bactéries, et ce indépendamment de la concentration en cuivre.

Si l'acide domoïque n'est pas produit pour protéger les cellules d'une forte dose de cuivre, il l'est peut être pour permettre aux cellules d'acquérir fer et cuivre. Ainsi, Wells et al. (2005) suggèrent que toutes les espèces de *Pseudo-nitzschia* sont potentiellement toxiques en cas de limitation en fer et/ou cuivre. Une espèce initialement non toxique dont la croissance est limitée par le fer et/ou le cuivre devrait donc produire de l'acide domoïque. C'est ce que nous avons testé dans les expériences suivantes, qui font l'objet du chapitre 6.

EFFETS DE LIMITATIONS EN FER ET/OU CUIVRE SUR *PSEUDO-NITZSCHIA DELICATISSIMA*

6



Article :

Lelong A., Bucciarelli E., Hégaret H., Soudant P. Iron and copper limitations impact growth rates, photosynthetic and physiological parameters of the marine diatom *Pseudo-nitzschia delicatissima*. En préparation pour soumission à Environmental Microbiology.

6.1. Préambule

Le fer intervenant dans de nombreux cycles cellulaires, tels la photosynthèse, la respiration et l'assimilation des nitrates (Sunda, 1988-1989), ses concentrations subnanomolaires dans 50 % l'Océan ouvert limitent la croissance optimale des espèces de microalgues présentes (de Baar et al., 2005, Boyd and Ellwood, 2010). Ce n'est pour autant pas le seul métal présent en concentration limitante dans certaines régions du monde. Dans une moindre mesure, le cuivre s'est récemment avéré aussi pouvoir limiter la croissance phytoplanctonique en mer de Béring (Peers et al., 2005). L'une des hypothèses actuelles voudrait que le cuivre ne soit pas directement limitant pour les cellules mais entraîne une limitation des cellules en fer (Maldonado et al., 2006). En effet, le cuivre est un composant des systèmes haute affinité de transport du fer (Maldonado et al., 2006). Certaines espèces de *Pseudo-nitzschia*, dont *P. delicatissima*, se retrouvent dans tous les océans mondiaux, en milieu côtier et océanique et ont donc réussi à s'adapter à ces concentrations variables et souvent limitantes en fer et cuivre. Ce sont aussi les diatomées de ce genre qui dominent souvent la communauté phytoplanctonique après un apport de fer à l'Océan ouvert (Coale et al., 2004, de Baar et al., 2005, Marchetti et al., 2006, Trick et al., 2010). L'objectif de ce chapitre est double. Pour commencer, nous avons voulu vérifier l'hypothèse de Wells et al. (2005) selon laquelle toute espèce de *Pseudo-nitzschia* limitée en fer peut être toxique. Si cette hypothèse est vraie et que l'acide domoïque est produit afin d'augmenter la biodisponibilité du fer et du cuivre, des limitations en ces micronutriments devraient induire la production d'acide domoïque chez *P. delicatissima*, ce qu'aucune des expériences précédentes n'a permis d'observer. D'autre part, nous avons voulu vérifier si le cuivre est principalement utilisé par ces cellules pour l'acquisition du fer ou si le cuivre a un rôle biologique indépendant du fer, induisant donc des modifications physiologiques différentes.

6.2. Article 6 - Iron and copper limitations impact growth rates, photosynthetic and physiological parameters of the marine diatom Pseudo-nitzschia delicatissima

Iron and copper limitations impact growth rates, photosynthetic and physiological parameters of the marine diatom *Pseudo-nitzschia delicatissima*

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Running head: Physiology of Fe- and Cu-limited *P. delicatissima*

Abstract

In ~ 50% of the ocean, iron (Fe) limits phytoplankton growth, and notably that of *Pseudo-nitzschia*. Fe-limited *Pseudo-nitzschia* spp. may thus produce the potent neurotoxin domoic acid (DA) to access Cu, needed at the core of a high affinity Fe transport system. We investigated here the growth, physiology and DA production of *P. delicatissima* under Fe limitations, Cu starvation and Fe/Cu co-limitations. Growth parameters confirmed that *P. delicatissima*, like other *Pseudo-nitzschia* spp., has a very low Fe requirement, and that Fe and Cu interplay in an essential manner. At the physiological level, Fe limitations decreased chlorophyll content and quantum yield (QY). Severe Fe limitation decreased esterase activity and storage of lipids while mild Fe limitation increased both. Cu starvation increased chlorophyll content, lipid content and esterase activity, with QY being identical to replete cells. Co-limitations induced modifications close to, but significantly different from Fe limitations. These results indicate that the Cu demand for Fe acquisition may be low relative to other cellular Cu pools and/or that this species may not use this high affinity Fe uptake system. This may explain why this species did not produce DA, even under mild Fe limitation with enough energy to do so.

Keywords: *Pseudo-nitzschia*, iron, copper, limitation, physiology

Introduction

Iron (Fe) is by far the most abundant metal in phytoplankton cells (Ho et al., 2003), with more than 50% of intracellular Fe located in the photosynthetic system, mainly in photosystems I and II (PSI and PSII) and cytochrome b_6-f (Strzepek and Harrison, 2004). Some superoxide dismutases and thylakoid components are also Fe-containing, and Fe plays a crucial role in the assimilation of nitrogen (Sunda, 1988-1989; Raven et al., 1999). In 50% of the surface waters of the open ocean, its sub-nanomolar concentrations are low enough to limit phytoplankton growth, with major implications for the biogeochemical cycles of carbon, nitrogen and silicon. Over the last 20 years, it has indeed been convincingly demonstrated that Fe enrichment of surface waters of the ocean, notably in the High Nutrient Low Chlorophyll areas, results in an increase of phytoplankton growth, especially diatoms (de Baar et al., 2005; Boyd and Ellwood, 2010). During Fe enrichment experiments, pennate diatoms of the genus *Pseudo-nitzschia*, many of which periodically produce the potent neurotoxin domoic acid (DA), often become dominant (Coale et al., 2004; de Baar et al., 2005; Marchetti et al., 2006b; Trick et al., 2010). Rue and Bruland (2001) showed that DA is a chelatant of Fe and copper (Cu), but the hypothesis that *Pseudo-nitzschia* produce DA in order to enhance Fe bio-availability is still debated. Indeed, DA production by Fe-limited *Pseudo-nitzschia* can increase (Maldonado et al., 2002; Trainer et al., 2009) or decrease (Bates et al., 2001) compared to Fe replete cells. DA was also found in higher concentrations after an *in situ* Fe enrichment than before (Silver et al., 2010; Trick et al., 2010).

Wells et al. (2005) hypothesized that *Pseudo-nitzschia* spp. actually produce DA to access Cu, which may play a key role in Fe acquisition. Cu is indeed at the core of a multicopper oxidase, an enzyme needed as part of the inducible Fe transport system of some Fe-limited phytoplanktonic cells (La Fontaine et al., 2002; Peers et al., 2005; Wells et al., 2005; Maldonado et al., 2006). Besides, some diatoms can replace Fe-rich cytochrome c_6 by the functionally equivalent Cu-containing plastocyanin (Peers and Price, 2006). To date only two Cu enrichment experiments of natural planktonic communities have been conducted, and they both showed that Cu indeed increases the growth of Fe-limited phytoplankton in surface waters of the open ocean (Coale, 1991; Peers et al., 2005). Coale (1991) explained the increase in phytoplankton growth of the subarctic Pacific after Cu enrichment by a decrease in microzooplankton grazing due to Cu toxicity. Peers et al. (2005), on the contrary, showed that Cu addition to phytoplankton from the Bering Sea increased phytoplankton biomass without

affecting zooplankton grazing rate. In culture experiments, Annett et al. (2008) however showed that Fe limitation increases cellular Cu quota in only two species over eight centric diatoms. They proposed that in certain species the Cu demand of the high-affinity Fe transport system may be low relative to other cellular Cu pools, like Cu-containing superoxide dismutase and cytochrome *c* oxidase.

Many studies have already been published on the effects of Fe limitation on diatoms (e.g. review by Sarthou et al., 2005; Marchetti and Cassar, 2009), including *Pseudo-nitzschia* (Maldonado et al., 2002; Wells et al., 2005; Marchetti et al., 2006a; Marchetti and Harrison, 2007). These studies mainly focused on growth parameters, photosynthetic efficiency, elemental composition, and DA production for *Pseudo-nitzschia*. On the contrary, the effects of Cu limitation on diatoms (Peers et al., 2005; Annett et al., 2008; Zhu et al., 2010), and especially on *Pseudo-nitzschia* (Wells et al., 2005) are still scarce.

Here we study how Fe and Cu limitations and co-limitations impact the growth rate and some of the morphological and physiological parameters of *Pseudo-nitzschia delicatissima*. All these measurements were performed in order to test, at the physiological level, (i) if there was an interaction between Fe and Cu, (ii) if the main effect of Cu limitation in this pennate marine diatom is to induce Fe limitation and (iii) how Fe and Cu could modify DA production. The photosynthetic parameters of the cells were studied, as Fe and Cu limitations are known to modify them (Inda et al., 1999; Moseley et al., 2000; Strzepek and Harrison, 2004). Photosynthesis and respiration-derived energy can be used for both primary metabolism (measured using esterase activity) and secondary metabolism. When cells have energy they do not, or cannot, use for primary and/or secondary metabolisms, they store it under lipid form (measured using a probe staining neutral lipids). DA is a secondary metabolite (Pan et al., 1998), thus produced when cells have more energy than needed for primary metabolism and growth. Measuring all these parameters can help understanding to which metabolism cells allocate their energy (Lelong et al., 2011). These measurements were performed using flow cytometry and pulse amplitude modulated fluorometry, and conducted for Fe and Cu replete cells, three degrees of Fe limitation, Cu starvation and two degrees of Fe/Cu co-limitation.

Results

Specific growth rate and morphological parameters

Regardless of the limitation, the growth rate decreased during the first culture transfer and then remained stable during the acclimation to the different conditions, i.e. there was no significant variation during more than 20 generations. The higher growth rate was observed for the control cultures (Table 1, Fig. 1, $\mu = 0.91\ d^{-1}$). The decrease in the specific growth rate with the total Fe concentrations was determined using a Monod saturation function (n = 12, $R^2 = 0.86$). It gives a maximum specific growth rate (μ_{max}) of $0.96 \pm 0.08\ d^{-1}$, and a half-saturation constant for growth with respect to total iron ($K_{\mu Fe}$) and inorganic iron ($K_{\mu Fe'}$) of 5.13 ± 1.51 nM and 3.1 ± 0.91 pM, respectively.

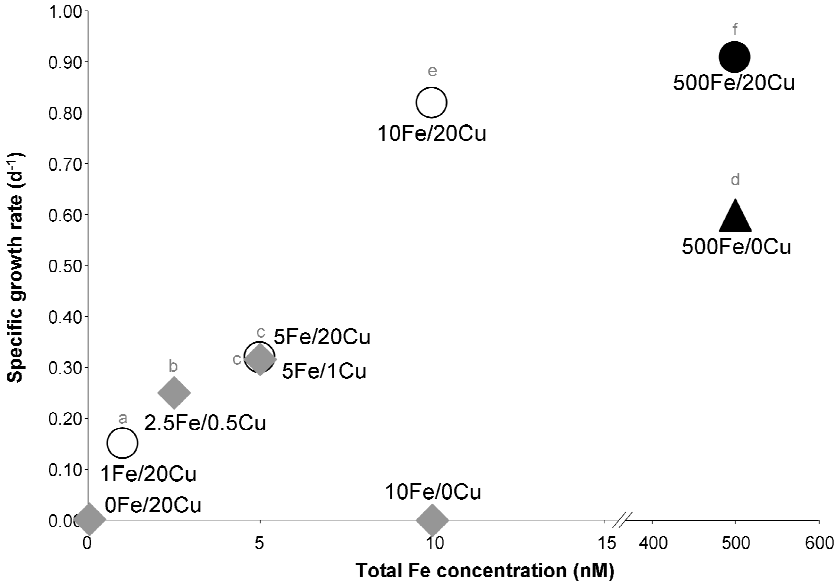

Fig 1. Specific growth rate of *P. delicatissima* versus total iron concentration in the medium for Fe limitation (open circles), Fe-Cu co-limitation (grey diamonds), Cu starvation (filled triangle), replete conditions (filled circle) (see Text/Table 1 for notation). Each symbol represents the mean, ± SE (n = 3) and letters represent different groups after one way ANOVA and Tukey rank test with $p < 0.05$.

0Cu and 10Fe cultures induced mild limitations ($\mu > 0.60\ \mu_{max}$), whereas 1Fe, 5Fe, 2.5Fe/0.5Cu, 5Fe/1Cu cultures induced severe limitations ($\mu < 0.35\ \mu_{max}$). Cultures did not grow under Fe starvation (0Fe) nor under Cu starvation when mildly Fe-limited (10Fe/0Cu). All the growth rates were significantly different ($p < 0.01$), except for 5Fe and 5Fe/1Cu cultures which grew at the same rate ($p > 0.05$, Fig. 1). However, all the other parameters (see below) indicated that 5Fe and 5Fe/1Cu cultures behaved differently at the physiological level, i.e. that 5Fe/1Cu cells experienced co-limitation.

Concerning morphological parameters, Cu-starved cells exhibited a significantly higher FSC and SSC than all the other cultures (Table 1). Using SSC, cell volume of Cu-starved cells was estimated to be ~ 25 % higher than that of replete cells. A 20 % increase in the cell volume of Cu starved cells was also observed by direct cell volume measurements in another experiment (Lelong, unpublished data). There was no clear trend for Fe-limited cells. Cells in 1Fe cultures had an estimated cell volume about 10 % lower than replete cells, whereas 10Fe cells had a 10 % higher estimated cell volume, but this may not be significant. Estimated cell volume for 5Fe cells, meanwhile, was about 40 % lower than in the control. When 5Fe cells were also Cu limited (5Fe/1Cu), however, and even though the growth rate remained similar, estimated cell volume significantly increased by about 25 %. The two co-limited cultures had the same SSC-estimated cell volume, which was about 20 % lower than the control (Table 1).

Photosynthetic parameters

The FL3 value, which can be related to the chlorophyll content of cells, and the quantum yield (QY), which is a measurement of the photosynthetic efficiency at the PSII level, were both measured. Fe-limited cultures exhibited significantly lower values of chlorophyll, chlorophyll production rate and QY than control cultures ($p < 0.01$, Fig. 2, Table 1). All these values decreased with the degree of Fe limitation, even when SSC-estimated cell volume was considered. On the contrary, Cu-starved cultures showed a significantly higher value of chlorophyll and chlorophyll production rate ($p < 0.01$) than control cultures. Even when taking into account the ~ 25 % increase in cell volume, Cu-starved cells seemed to contain about 40 % more chlorophyll than replete cells. The QY, however, was the same in Cu-starved cultures and in the control ($p > 0.05$). Co-limited cultures also seemed to have significantly more chlorophyll, and as a result a higher chlorophyll production rate, and a higher QY than Fe-limited cultures at an equivalent growth rate (e.g. 5Fe and 5Fe/1Cu cultures), even when SSC-estimated cell volume was considered.

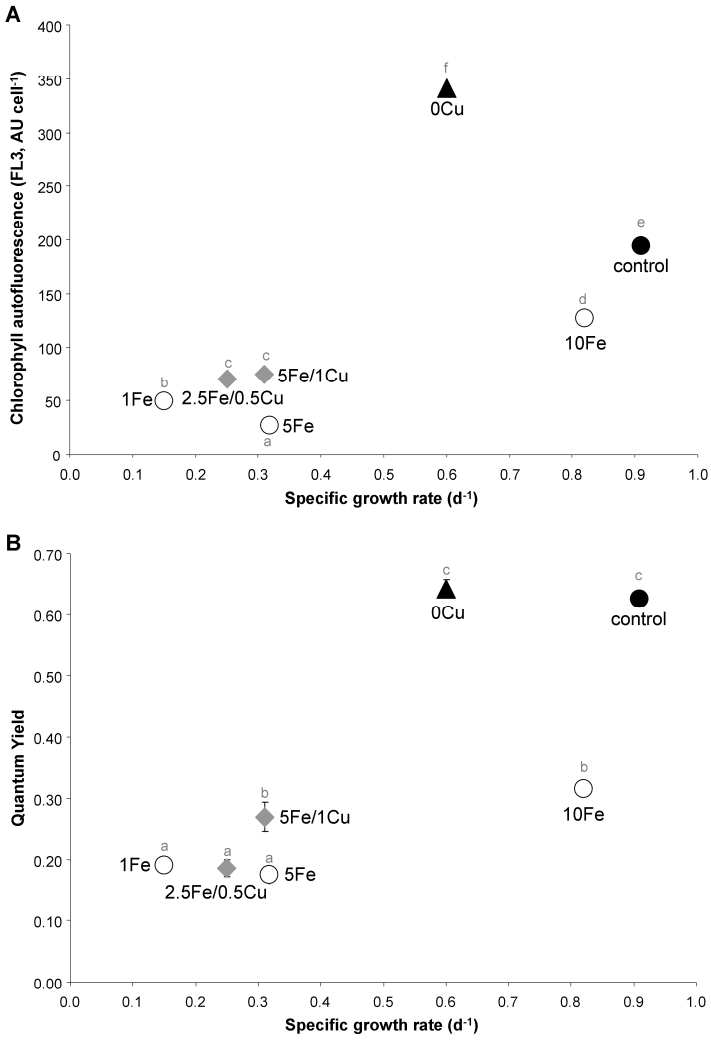

Fig 2. A. Chlorophyll autofluorescence and B. Efficiency of photosynthesis (Quantum Yield, QY) of *P. delicatissima* versus specific growth rate for Fe limitation (open circles), Fe-Cu co-limitation (grey diamonds), Cu starvation (filled triangle), replete conditions (filled circle) (see Text/Table 1 for notation). Each symbol represents the mean, ± SE (n = 3) and letters represent different groups after one way ANOVA and Tukey rank test with $p < 0.05$.

Physiological measurements

Esterase activity (i.e. activity of primary metabolism) under Fe limitation decreased only for the most limited cells (1Fe), per cell (Fig. 3A) and per cell volume. Cu-starved cells exhibited higher esterase activity than replete cells, both per cell and per cell volume ($p < 0.01$). When both limitations applied, however, co-limited cells had lower esterase activity per cell and per cell volume than control ($p < 0.01$).

Severe Fe limitations did not modify the lipid content per cell (Fig. 3B), but caused its increase per cell volume. However, when bio-dilution was taken into account, severely Fe-limited cells produced less lipid than replete cells. Mild limitation, on the contrary, by either

Fe or Cu, increased both production rate and intracellular content, per cell and per cell volume, whereas lipid content (per cell and per cell volume) decreased under Fe-Cu co-limitation.

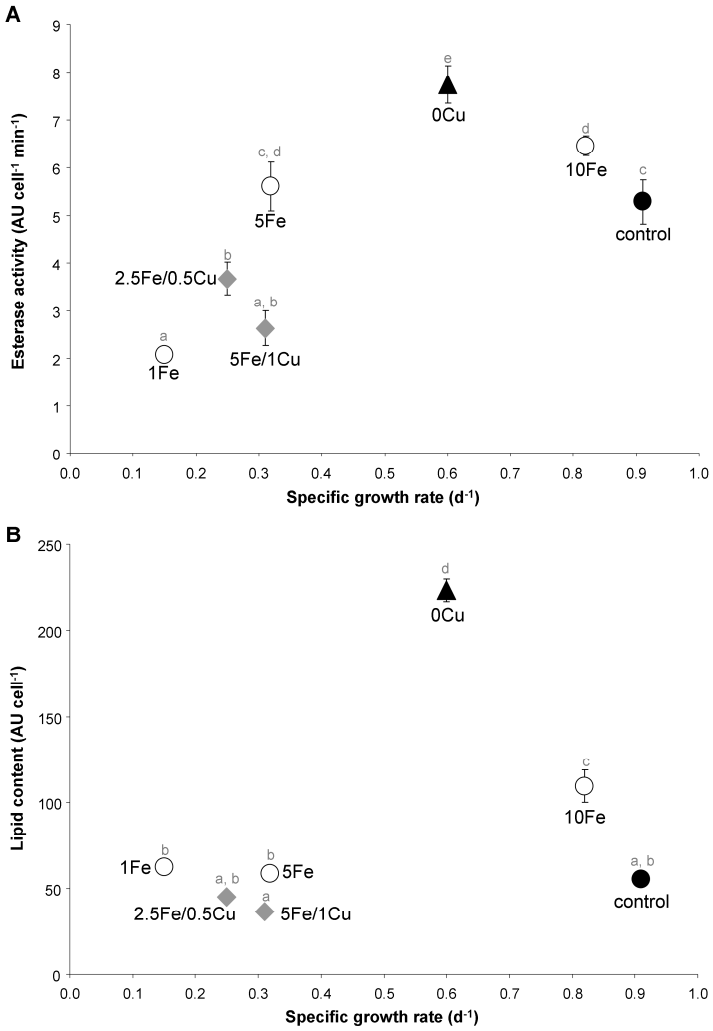

Fig 3. A. Esterase activity and B. Lipid content of *P. delicatissima* versus specific growth rate for Fe limitation (open circles), Fe-Cu co-limitation (grey diamonds), Cu starvation (filled triangle), replete conditions (filled circle) (see Text/Table 1 for notation). Each symbol represents the mean, ± SE (n = 3) and letters represent different groups after one way ANOVA and Tukey rank test with $p < 0.05$.

Domoic acid and bacteria

None of our cultures produced detectable level of DA, regardless of the limitation. All of the cultures contained free-living bacteria, and their survival was not modified by culture conditions (i.e. Fe and/or Cu limitations).

Table 1: Specific growth rate (µ, d^{-1}), morphological parameters (FSC, SSC, cell volume estimated from SSC values), photosynthetic parameters (chlorophyll content and production rate, Quantum Yield), and physiological parameters (esterase activity, intracellular lipid content and production rate) of *P. delicatissima* (mean, SE, n = 3) for replete cells (control), Fe-limited cells (1Fe, 5Fe and 10Fe), Cu-starved cells (0Cu) and co-limited cells (2.5Fe/0.5Cu, 5Fe/1Cu). AU = arbitrary units.

	Replete			Fe limitations							Cu starvation			Co-limitations					
Notation	Control			1Fe			5Fe			10Fe			0Cu			2.5Fe/0.5Cu			5Fe/1Cu		
Total Fe/Cu concentration (nM)	500Fe/20Cu			1Fe/20Cu			5Fe/20Cu			10Fe/20Cu			500Fe/0Cu			2.5Fe/0.5Cu			5Fe/1Cu		
	mean	SE		mean	SE		mean	SE		mean	SE		mean	SE		mean	SE		mean	SE	
µ (d^{-1})	0.91	0.01	f	0.15	0.01	a	0.32	0.01	c	0.82	0.00	e	0.60	0.00	d	0.25	0.00	b	0.31	0.01	c
FSC (AU)	41.5	0.8	c	29.9	0.4	b	24.2	0.2	a	52.4	3.6	d	89.9	0.5	e	29.6	0.3	b	31.3	0.5	b
SSC (AU)	12.7	0.2	d	11.6	0.1	c	8.1	0.1	a	14.1	0.2	e	16.0	0.1	f	10.3	0.1	b	10.2	0.1	b
SSC-estimated cell volume (μm^3)	103		d	94		c	65		a	114		e	130		f	83		b	82		b
Chlorophyll (AU $cell^{-1}$)	194.2	3.6	e	49.0	0.3	b	27.2	0.3	a	126.5	5.9	d	341.2	0.7	f	70.4	0.2	c	74.4	1.3	c
Chlorophyll production rate (AU $cell^{-1}$ d^{-1})	176.2	5.2	e	7.5	0.5	a	8.7	0.4	a, b	103.7	4.8	d	204.7	0.4	f	17.6	0.1	b, c	23.1	1.2	c
Quantum Yield	0.62	0.01	e	0.19	0.02	a	0.17	0.04	a, b	0.32	0.01	d	0.64	0.02	f	0.19	0.02	b, c	0.27	0.03	a, b
Esterase activity (AU $cell^{-1}$ min^{-1})	5.3	0.5	c	2.1	0.1	a	6.2	0.5	c, d	6.5	0.2	d	7.8	0.4	e	3.7	0.3	b	2.6	0.4	a, b
Intracellular lipid (AU $cell^{-1}$)	55.4	1.3	a, b	62.6	0.8	b	58.9	0.7	b	109.5	9.4	c	223.6	6.4	d	45.2	0.9	a, b	36.9	1.2	a
Lipid production rate (AU $cell^{-1}$ d^{-1})	50.4	1.7	b	9.4	0.8	a	18.9	0.8	a	89.8	7.7	c	134.2	3.8	d	11.3	0.2	a	11.4	0.7	a

Table 2: Maximum specific growth rate (µmax, d^{-1}), half-saturation constants for growth with respect to total iron ($K_{\mu Fe}$) and to inorganic iron ($K_{\mu Fe'}$) and cell volume of different species of *Pseudo-nitzschia*.

Genus	Habitat	Species	Reference	μ_{max} (d^{-1})	$K_{\mu Fe}$ (total Fe, nM)	$K_{\mu Fe'}$ (inorganic Fe, pM)	Cell volume (μm^3)
Pseudo-nitzschia	Coastal	*P. delicatissima*	This study	0.96 ± 0.08	5.13 ± 1.51	3.1 ± 0.91	100
		P. australis	Maldonado et al., 2002	0.80 ± 0.24	~ 1.7	~ 8.7	4 750
		P. multiseries	Maldonado et al., 2002	1.00 ± 0.17	~ 1.7	~ 8.7	1 270
			Marchetti et al., 2008	~ 1.6	~ 10	~ 6	-
	Oceanic	*P. granii*	Marchetti et al., 2008	~ 1.6	~ 1.8	~ 1.1	968
		P. heimii type 1	Marchetti and Harrison, 2007	~ 1.4	~ 3.1	~ 1.9	-

Discussion

Growth parameters under Fe and Cu limitations

All our cultures grown under limiting Fe and/or Cu exhibited a decrease of their growth rate, indicating that the cultures were experiencing severe to mild limitations (Fig. 1).

A decrease in the specific growth rate of phytoplankton species, including diatoms, is generally observed when Fe limitation is achieved (e.g. Sunda and Huntsman, 1995; Sarthou et al., 2005). This is also observed for *Pseudo-nitzschia* species in most cases (Maldonado et al., 2002; Marchetti et al., 2006a; Marchetti and Harrison, 2007; Marchetti et al., 2008), although adaptation to Fe limitation through domoic acid (DA) production may help some of the species to achieve maximum growth rates after numerous transfers (Wells et al., 2005). Marchetti et al. (2008) also studied the specific growth rates of *P. multiseries* and *P. granii* at different degrees of Fe limitation, which allows calculating a maximum specific growth rate and half-saturation constants for growth with respect to total iron and to inorganic iron (taking into acccount the EDTA concentration in their medium, see *Culture media* in Experimental procedures) (Table 2). Maldonado et al. (2002) and Marchetti and Harrison (2007) grew *P. multiseries*, *P. australis* and *P. heimii* type 1 at only one Fe-limiting concentration, but it decreased by half the growth rate compared to replete cells, i.e. the total Fe concentration they used can be considered close to $K_{\mu Fe}$ for these species, and $K_{\mu Fe'}$ can be estimated. Comparing $K_{\mu Fe'}$ of these different *Pseudo-nitzschia* species indicates that *P. multiseries* and *P. australis* are 2- to 3-fold more sensitive to iron depletion than *P. delicatissima*, while *P. granii* and *P. heimii* type 1 exhibit the best adaptations (Table 2). The difference between these species can be partially explained by their habitat. Indeed, oceanic species are more adapted to low Fe concentration than coastal species (Sunda et al., 1991; Sunda and Huntsman, 1995; Marchetti et al., 2006a). Cell size may also explain the differences in Fe requirement between these coastal species. The cell volume of *P. delicatissima* is indeed much smaller than those of *P. multiseries* and *P. australis* (Table 2), i.e. the surface-to-volume ratio is higher, which helps acquiring limiting nutrients like Fe (Hudson and Morel, 1990). Maldonado et al. (2002) also noted that at similar Fe limiting concentrations, *P. multiseries* and *P. australis* grew faster relative to their maximum specific growth rate (μ/μ_{max}) than other coastal diatoms.

Maldonado et al. (2002) hypothesized that the release of DA, which is a ligand with high affinity for Fe in seawater (Rue and Bruland, 2001), may explain the competitive advantage of *Pseudo-nitzschia* cells. This hypothesis, however, does not hold for our non toxic strain of

P. delicatissima. One of the other adaptative mechanisms that allows Fe-limited cells to better acquire limiting nutrients is a decrease in cell size, which is commonly observed under Fe limitation (e.g. Sunda and Huntsman, 1995). In our case however no consistent trend was observed in cell size variations of *P. delicatissima* at different degrees of Fe limitation. Other adaptative mechanisms to Fe limitation include the replacement of Fe-rich molecules by some containing either no metal or another metal, e.g. ferredoxin by flavodoxin (La Roche et al., 1993), or Fe-superoxide dismutase (SOD) by Mn -SOD (Peers and Price, 2004). However, because phytoplankton cells need ~ 10 times more Fe than any other trace metal, it is unlikely for Fe-limited cells to substitute all Fe-containing molecules by functionally equivalent molecules using other metals (Ho et al., 2003). Thus, even though *P. delicatissima* (and *Pseudo-nitzschia* spp. in general) seems to have very low Fe requirements compared to other diatoms, it still has an absolute requirement for this nutrient as evidenced by the zero growth rate of Fe-starved cultures. On the opposite, Cu starvation only induced a mild limitation (66 % of μ_{max}). This decrease of growth rate has already been observed for Cu-starved *Thalassiosira weissflogii* and Cu-limited *T. oceanica* and *P. fraudulenta* (Wells et al., 2005). Experiments run on Cu-limited *P. multiseries* and *P. australis* showed however no decrease of growth rate compared to control cultures (Wells et al., 2005). Our experiments also show that for this species, Fe and Cu seem to interplay in an essential manner. Indeed, even though Cu starvation only induced a mild limitation in *P. delicatissima*, this species has nonetheless an absolute requirement for Cu to grow under Fe limitation, as indicated by the zero growth rate under mild Fe limitation and Cu starvation (10 nM of Fe and no Cu). This essential Cu requirement to grow under Fe limitation seems however low (< 1nM of total Cu) as evidenced by the cell ability to (i) maintain the same growth rate under severe Fe limitation (5 nM of Fe) at 1 nM and 20 nM Cu, and (ii) to grow under severe Fe limitation and severe Cu limitation (2.5 nM of Fe and 0.5 nM of Cu). Annett et al. (2008) found that co-limited cultures of six different coastal species of diatom had the same low growth rate when co-limited than when only Fe-limited. Conversely, Zhu et al. (2010) found that *Thalassiosira pseudonana* grew more slowly when co-limited than when only Fe-limited. Similar observations to Zhu et al. (2010) have also been made for *P. australis*, *P. multiseries* and *P. fraudulenta* (Wells et al., 2005).

It was first hypothesized that Fe-limited phytoplankton had an increased need of Cu because of the use of a multi-copper oxidase as part of a high-affinity Fe transport system (Wells et al., 2005). It was later proved that Cu limitation indeed decreased Fe uptake rates, and that

transcription levels of a putative multi-copper oxidase gene were significantly elevated in Fe-limited cells of *T. pseudonana* (Peers et al., 2005; Maldonado et al., 2006). More recent studies, however, demonstrated that for some diatoms, the Cu demand of the high-affinity Fe transport system may be low relative to other cellular Cu pools, like Cu-containing SOD and cytochrome *c* oxidase (Annett et al., 2008). For example, although the oceanic diatom *T. oceanica* needs Cu to acquire Fe (Peers et al., 2005), most of the Cu requirement of this species is attributable to its use of the Cu-containing plastocyanin rather than Fe-rich cytochrome c_6 in the photosynthetic apparatus (Peers and Price, 2006). To further explore if the main effect of Cu limitation in *P. delicatissima* was to induce Fe limitation, we compared the effects of Fe and Cu limitations on photosynthetic parameters, activity of primary metabolism and lipid content.

Does Cu limitation induce Fe limitation at the physiological level in *P. delicatissima*?

One of the well known impacts of Fe limitation is the decrease of photosynthetic abilities. Indeed, Fe is a major component of the photosynthetic chain reaction of microalgae; with twelve atoms in photosystem I (PSI), five in cytochrome b_6-*f*, two to three in photosystem II (PSII), two in ferredoxin and one in cytochrome c_6 (Raven et al., 1999). Chlorophyll autofluorescence is assumed to be proportional to chlorophyll (chl) content and the decrease of chl *a* content (or chlorosis) has been previously shown to be a proxy of Fe limitation (Greene et al., 1992). This decrease in chl *a* content has already been demonstrated for Fe-limited *Pseudo-nitzschia* sp. (Bates et al., 2001; Marchetti and Harrison, 2007). In the present study, not only did chl *a* content decrease, but quantum yield (QY) also decreased. The QY measures the maximum photochemical yield of PSII, thus the efficiency of photosynthesis at the beginning of the photosynthetic chain. Under Fe limitation, cells have less PSI and PSII, in an extent depending on the species (Strzepek and Harrison, 2004). The decrease of QY has been previously shown for different species of *Pseudo-nitzschia* during or after adaptation to low Fe concentration (Wells et al., 2005; Marchetti and Harrison, 2007; Marchetti et al., 2008). The comparison of the photosynthetic efficiency of different *Pseudo-nitzschia* spp. under Fe limitation shows that all species decrease their QY under Fe limitation, but this decrease is not always related to a decrease of growth rate (Fig. 4), i.e. some species can maintain their growth in spite of impaired photosynthesis, to more than 90 % of μ_{max} (Marchetti and Harrison, 2007; Marchetti et al., 2008).

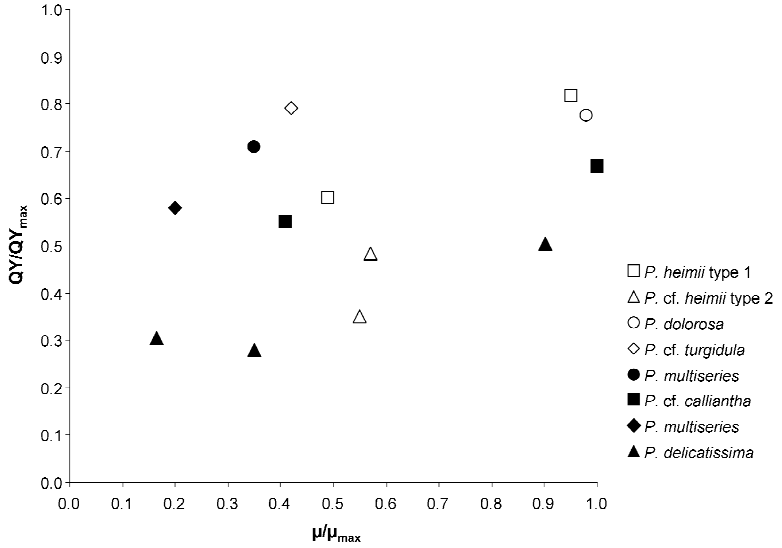

Fig. 4. Relative variation of photosynthesis efficiency (QY/QY_{max}) versus relative variation in specific growth rate (μ/μ_{max}) under Fe limitation for *P. delicatissima* (this study), *P. heimii* type 1 (open square, Marchetti and Harrison, 2007), *P.* cf. *heimii* type 2 (open triangle, Marchetti and Harrison, 2007), *P. dolorosa* (open circle, Marchetti and Harrison, 2007), *P.* cf. *turgidula* (open diamond, Marchetti and Harrison, 2007), *P. multiseries* (closed circle, Marchetti et al., 2008, and closed diamond, Marchetti and Harrison, 2007) and *P.* cf. *calliantha* (closed square, Marchetti and Harrison, 2007). Open symbols are oceanic species and closed symbols are coastal species.

On the opposite, *P. delicatissima* cells under Cu starvation maintained a QY identical to replete cells but exhibited an increase of their chlorophyll content (as shown by increased FL3 values), even when cell volume was considered. Zhu et al. (2010) found that Cu limitation did not modify chl *a* content of *T. pseudonana* and La Fontaine et al. (2002) did not find any chlorosis in Cu-limited *C. reinhardtii* cells either. For some microalgal species, Cu limitation can induce a shift from plastocyanin to cytochrome c_6 in *T. weissflogii* and *C. reinhardtii* (Inda et al., 1999; Moseley et al., 2000). Plastocyanin, a Cu-containing protein between cytochrome b_6-*f* complex and PSI was found in *T. oceanica* in replacement of cytochrome c_6 (Peers and Price, 2006). However, although our results clearly demonstrate that *P. delicatissima* was able to maintain an efficient photosynthesis at the PSII level, there is no proof that this adaptation occurs through plastocyanin / cytochrome c_6 ratio adjustment or even that the photosynthetic apparatus was fully functional. QY indeed only takes into account PSII efficiency, and would not be affected by a modification of Cu-containing proteins at a later stage of the photosynthetic chain (Peers and Price, 2006). Other proteins and structural components may also be involved in such an adaptation. Under different stresses, microalgae are able to modify the lipid composition of their thylakoid membrane in order to maintain the functional integrity of photosynthetic membranes (Mock and Kroon, 2002a, b). Such a modification of the lipid composition could modify light absorbance

(Ventrella et al., 2007) and thus cell autofluorescence and explain the increase in FL3 observed in this study. Although it can be noted that lipid content varied greatly between Cu-starved cells and replete cells, lipid composition, as well as photosynthetic pigments need to be fully characterized and quantified to better understand the maintenance of photosynthetic efficiency at the PSII level and the seemingly increase in chlorophyll under copper starvation. Regardless of the mechanisms at play, it can nonetheless already be asserted that Cu limitation (either starvation or co-limitation) had opposite effects on photosynthetic parameters than Fe limitation, strongly suggesting that the main effect of Cu limitation is not to induce Fe limitation in *P. delicatissima.* Fe and Cu however interplay at the physiological level, as evidenced by the fact that cells are not able to survive mild Fe limitation when also Cu-starved.

Fe and Cu are not only components of the photosynthetic chain, but also of many other proteins, such as for example superoxide dismutases (SOD). Under Fe limitation, Fe-SOD can be replaced by Mn-SOD (Peers and Price, 2004). Changes in proportion of these different isoforms of SOD may affect metal cell demand and cellular physiology. Fe and Cu are also involved in essential processes that are not part of the 'core' non-cyclic electron flow. These processes include the NADPH dehydrogenase which is also involved in chlororespiration and cyclic electron flow around PSI (Raven et al., 1999). Fe and Cu are also components of the respiratory chain (Falkowski and Raven, 1997). Under Fe limitation, respiration has been shown to be down regulated in microalgae (Allen et al., 2008; Petroutsos et al., 2009). Because Cu is also a component of the respiratory chain, Cu limitation may also down regulate respiration.

As a result, Fe and Cu limitations should affect both primary and secondary metabolisms, which depend on energy acquired through photosynthesis and respiration. Primary metabolism was estimated here through esterase activity. Cells undergoing mild limitation (10Fe and 0Cu) had a higher esterase activity than control cells, while cells undergoing severe limitation (both Fe-limited and Fe/Cu co-limited) reduced their esterase activity (except for 5Fe cultures). When cells have extra energy that they are not able to use for primary metabolism (including growth), they store it under lipid form, at the expense of protein or carbohydrate synthesis (Giordano et al., 2001). This relationship is readily visible on figure 5 which clearly shows that lipid production rate increased with increasing esterase activity, regardless of the limitation. An important feature displayed by this figure is the difference in

the efficiency of lipid production relative to the activity of primary metabolism between severe and mild limitations. At the most severe limitations ($\mu < 35\ \%\ \mu max$), ratio of lipid production over esterase activity was almost constant ($2.6*10^{-3} \pm 0.4*10^{-3}$ AU AU^{-1}) and 2.5-fold lower than for replete cells ($6.6*10^{-3} \pm 0.8*10^{-3}$ AU AU^{-1}). As photosynthesis (and probably respiration) was lowered under severe limitation, it is likely that all available energy was used, none being stored under lipid form. Lipid content measured for these cells thus probably mainly represents structural lipids, which production can not be downregulated below ~10 AU $cell^{-1}$ d^{-1}. Mildly limited cells (10Fe and 0Cu), on the contrary, could increase this ratio up to $11.9*10^{-3} \pm 1.0*10^{-3}$ AU AU^{-1}, with production rate as high as 134 AU $cell^{-1}$ d^{-1}. Carvalho et al. (2006) also showed that Cu-limited cultures of *Pavlova lutheri* exhibited an increase of their lipid content. Because growth is reduced while photosynthesis does not seem to be affected, Cu-starved cultures may have extra energy to store under lipid form. Mildly Fe-limited cells (10 Fe) may also have had an imbalance between available energy and reduced growth rate which increased storage of lipids, even with reduced photosynthesis. On the contrary, replete cells did not need to store reserve lipids because they could use all their energy for growth. Thus lipid-related fluorescence using BODIPY, in control cells, likely corresponds to the staining of structural (membrane) lipids. A complete characterization and quantification of the different classes of lipids under different degrees of limitations would thus clearly improve our understanding of not only photosynthetic efficiency but also of how energy is distributed within primary and secondary metabolisms.

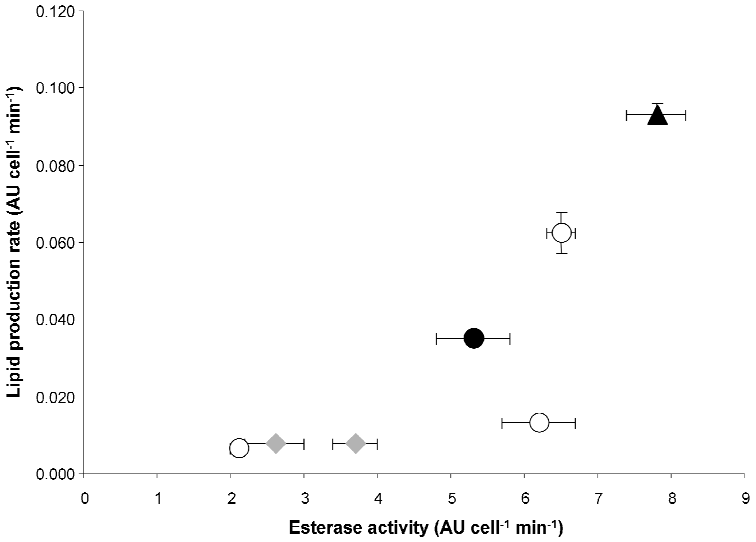

Fig. 5. Lipid production rate versus esterase activity for Fe limitation (open circles), Fe-Cu co-limitation (grey diamonds), Cu starvation (filled triangle), replete conditions (filled circle) (see Text/Table 1 for notation).

Finally, a discriminant analysis (Fig. 6) was run to group all physiological parameters. It allowed a discrimination of all the cultures, with 100 % of the samples well assigned to their group. The two main functions explained 71 and 23 % of the variance. Gradients in Fe and Cu limitations from severe limitation to replete cells were particularly evident on function 1 for Fe limitations and on function 2 for Cu limitations. This confirms that Cu limitation induced different physiological modifications from Fe limitations, i.e. that for *P. delicatissima*, most of cellular Cu is probably used for other physiological mechanisms than Fe transport. Annett et al. (2008) also found that for six out of eight strains (and five out of six species) Cu demand of the high-affinity Fe transport system is low relative to other cellular Cu pools. The discriminant analysis also suggests that during co-limitations, even though Cu had an effect by itself, Fe limitation triggered the main response. This is in agreement with the fact that Fe is more essential for cells than Cu (Ho et al., 2003; Zhu et al., 2010).

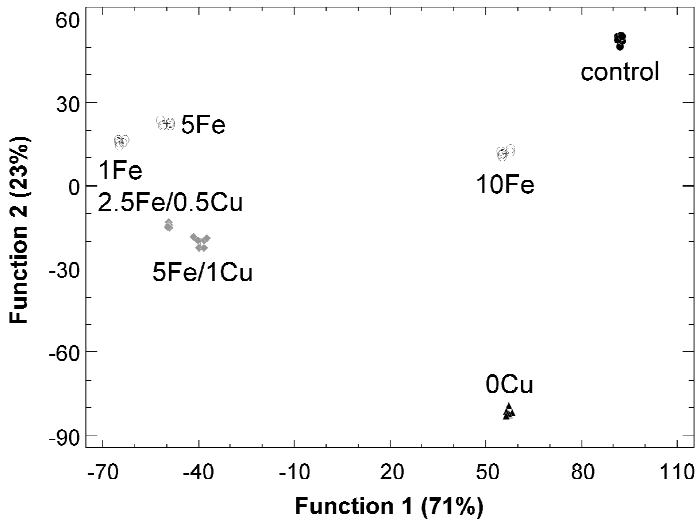

Fig 6. Discriminant analysis of *P. delicatissima* cultures grouping all physiological parameters for Fe limitation (open circles), Fe-Cu co-limitation (grey diamonds), Cu starvation (filled triangle), replete conditions (filled circle) (see Text/Table 1 for notation).

Impacts of Cu and Fe on the physiological processes of the cells and the hypothesis of DA production

The strain of *P. delicatissima* used in the present study was not able to produce DA, as some other *P. delicatissima* strains (Fehling et al., 2005; Thessen et al., 2005). DA is a secondary metabolite, produced when cells have enough energy besides that required for cell maintenance processes. We can thus hypothesize that highly limited cells, which were not able to grow, store any lipids or produce any esterase, probably would not have enough energy to produce any DA. On the opposite, mildly limited cells, with enough energy to store

lipids and increase esterase activity would definitely have energy to allocate to DA production if needed. Because DA is a Fe (and Cu) ligand in seawater, it was first hypothesized that Fe-limited *Pseudo-nitzschia* produced DA in order to chelate and uptake this micronutrient (Rue and Bruland, 2001). Indeed, some Fe-limited *Pseudo-nitzschia* (generally growing at 50 % μ_{max} or more, i.e. not being severely limited) produce more DA than replete cells, and adding DA either to the culture medium or in natural seawater increases their Fe uptake rates (Maldonado et al., 2002; Wells et al., 2005). However, the conditional stability constant between DA and Fe being low compared to other organic ligands in seawater, such as bacteria-excreted siderophores, DA concentrations as high as 100 nM would be needed for DA to effectively compete with siderophores (Rue and Bruland, 2001). As pointed out by Wells et al. (2005), these concentrations are rarely achieved in seawater. This and other direct observations led Wells et al. (2005) to hypothesize that DA is actually produced by Fe-limited diatoms to chelate and acquire Cu, which is needed as part of a high affinity Fe uptake system to ultimately access Fe complexed by stronger organic ligands like siderophores. Because Cu limitation induced different physiological responses from Fe limitation in our strain of *P. delicatissima*, one hypothesis is that this species does not use this high affinity Fe uptake system, and as such does not produce any DA even under mild Fe limitation. Its half-saturation constant for growth with respect to Fe compared to other diatoms being low (see Text and Table 2), it must have, however, an adaptative mechanism to decrease its cellular Fe requirements and/or increase Fe availability. Another hypothesis regarding DA production may also be postulated, that if DA-acquired Cu is used to access Fe complexed by siderophores (Wells et al., 2005), DA production may actually be triggered by a threshold concentration of siderophores in the medium. It has already been postulated that DA production could be triggered by gluconolactone/gluconic acid, produced by some bacteria as a metal chelator (Osada and Stewart, 1997; Stewart et al., 1997; Stewart, 2008). Cultures of *Pseudo-nitzschia* grown without bacteria indeed lose their toxicity (Douglas et al., 1993; Bates et al., 1995). Our Fe-limited cultures were not axenic, but the bacterial concentration might have been too low to trigger DA production. Moreover, bacterial community was modified by Fe and Cu limitations (data not shown) and DA production can be differently modulated by different bacterial community (Kaczmarska et al., 2005). Thus the link between Fe, Cu, DA production and bacteria still needs to be further investigated, especially with seemingly non toxic *Pseudo-nitzschia* species.

Experimental procedures

Culture conditions

Batch cultures of the marine pennate diatom *Pseudo-nitzschia delicatissima* (strain Pd08RB, solitary species isolated in 2008 by Beatriz Beker in the Bay of Brest, France, ca. 100 μm^3) were grown at 16°C in polycarbonate bottles. Species was determined after sequencing of the ITS-1 fragment, using PnAllR and PnAllF primers (Hubbard et al., 2008) and obtained sequences were aligned using GenBank. Cultures were grown under cool-white light (OSRAM) over a dark:light cycle of 12:12 h with an irradiance of 130 µmol photons m^{-2} s^{-1}. Although the cultures were naturally xenic and grown without antibiotic, the culture media (see below) were sterilized using micro wave (Keller et al., 1988). Diatoms were pre-acclimated to each culture condition until their growth rate remained stable over several days, and grown in triplicates. When analyses were conducted, at least 20 generations were grown in the same conditions and at an equivalent growth rate. Before each sampling, cultures were homogenized by 50 reversals. Cultures were sampled at mid-exponential phase of growth to maintain a pH lower than 8.5 and avoid CO_2 limitation, and at the same time to avoid diel cycle variations.

Culture media

The medium consisted of artificial AQUIL seawater enriched with 300 µM nitrate, 10 µM phosphate, 100 µM silicate, 0.55 µg l^{-1} vitamin B_{12}, 0.5 µg l^{-1} biotin, 100 µg l^{-1} thiamin, 10 nM selenite and 100 nM molybdate (Price et al., 1988/1989). The medium also contained a trace metal ion buffer system consisting of 100 µM ethylene diamine tetra acetic acid (EDTA), 50.3 nM Co, 79.7 nM Zn and 121 nM Mn. The buffer system generated free ion concentrations of Co, Zn and Mn of $10^{-10.88}$, $10^{-10.88}$ and $10^{-8.27}$ M, respectively, at pH 8.1 (Price et al., 1988/1989). In the replete medium, 500 nM Fe and 19.6 nM Cu were added. In the Fe-limited experiments, Fe additions were 0 (no addition), 1, 5, and 10 nM. In the Cu starvation experiment, no Cu was added to the medium. Three co-limited experiments were also conducted, containing 2.5 nM Fe / 0.5 nM Cu, 5 nM Fe / 1nM Cu and 10 nM Fe / 0 nM Cu. In the following manuscript, the cultures are referred to according to the limiting Fe or Cu concentrations in the medium in case of a single limitation (e.g. 1Fe, 0Cu,...), and with both values in case of a co-limitation (e.g. 2.5Fe/0.5Cu).

Background concentrations of Fe or Cu in the medium without EDTA were not measured. However in a previous study, following the same protocol for the preparation of the medium,

the background Fe concentration was 0.6 nM (Bucciarelli et al., 2010). All bottles and apparatus were acid cleaned, and all manipulations were conducted within a sterile laminar flow hood equipped with a Teflon® bench using sterile and trace-metal clean techniques (Bucciarelli et al., 2010). In these media, most of Fe is chelated to EDTA as the non biovailable complex Fe-EDTA, which maintains picomolar concentrations of bioavailable inorganic Fe (Fe'). To allow comparisons between studies with different EDTA concentrations (see Discussion), [Fe'] must be calculated. To do so, equations between the different forms of Fe in the medium ([Total Fe] = [Fe-EDTA] + [Fe']) and the equilibrium constant between EDTA and Fe (C = [Fe-EDTA] / ([Fe'] * [EDTA]) are solved using a value of $10^{7.18}$ for C (Sunda et al., 2005).

Specific growth rate, morphological and physiological parameters

Cell concentrations, morphological and physiological measurements, quantification of bacteria associated to *P. delicatissima* and percentage of dead bacteria in the culture were assessed using flow cytometry according to Lelong et al. (2011). A flow cytometer FACScalibur (BD Biosciences, San Jose, CA USA) with an argon blue laser (488 nm) was used, with the same settings for all the duration of the experiment to allow comparison between days. Each triplicate of culture was analyzed with technical duplicates. All the results obtained from flow cytometry are in arbitrary units of fluorescence and expressed per cell (for chlorophyll-related FL3, lipid-related BODIPY) and per cell per minute (for FDA-related esterase activity).

Specific growth rate and morphological parameters

Specific growth rate (μ, d^{-1}) was determined by linear regression of the natural log(cell concentration) versus time. The maximum specific growth rate (μ_{max}) and the half-saturation constant for growth with respect to iron ($K_{\mu Fe'}$) were calculated using a Monod saturation function. Forward Scatter (FSC, light scattered at less than 10 degrees) and Side Scatter (SSC, light scattered at a 90 degree angle) flow cytometric measurements were also measured to provide light diffraction related morphological information.

In 7 other experiments on Fe- and Cu- limited *P. delicatissima*, values of FSC and SSC were also measured by flow cytometry while 50 randomly selected cells were digitized on an inverted microscope using an analogic Leica camera and analyzed with software image analysis (Visilog 5) to determine their maximal width and length (Boutorh and Moriceau,

unpubl. data). The average cell volume was calculated using the geometric formula for an ellipsoid. Cell volume was well correlated to SSC (V_{cell} = 8.22 * SSC - 1.56, n = 7, R^2 = 0.92, $p < 0.01$) and data were more evenly distributed than when correlated to FSC (V_{cell} = 1.28 * FSC - 12.2, n = 7, R^2 = 0.88, $p < 0.01$). In our study, SSC was thus used to estimate cell volume.

Photosynthetic parameters

FL3 fluorescence (red fluorescence at 670 nm) can be related to the chlorophyll content of cells. Quantum yield ($QY=(F_m-F_0)/F_m=F_v/F_m$), which is a measurement of the efficiency of the photosynthesis at the photosystem II (PSII) level, was measured using the AquaPen-C AP-C 100 fluorometer (Photo Systems Instruments, Czech Republic) where F_0 and F_m are respectively the minimum and maximum fluorescence of cells at 455 nm. The measurement of QY was performed after 20 min of dark adaptation of the cells at 16°C.

Physiological measurements

Mortality of *P. delicatissima* was assessed by staining cultures with 0.1 µM of SYTOX Green (Molecular probes, Invitrogen, Eugene, Oregon, USA) for 30 minutes. It showed more than 95% of living cells in all of our cultures, which ensures that the physiological measurements were performed on living cells. Esterase activity, a proxy for the activity of primary metabolism, was assessed after staining the cells with 3 µM of fluorescein di-acetate (FDA, Molecular probes, Invitrogen, Eugene, Oregon, USA) for 6 minutes. A work solution of FDA at 300 µM was freshly prepared before each experiment. Uptake of FDA is time-dependent, as fluorescein is accumulated inside cells, thus results are expressed as arbitrary units per cell per minute (AU $cell^{-1}$ min^{-1}). Intracellular lipid content was assessed by staining lipids with 10 µM of BODIPY 493/503 (Molecular probes, Invitrogen, Eugene, Oregon, USA) for 30 minutes.

Domoic acid

Domoic acid (DA) content was quantified using ASP ELISA kit (Biosense Laboratories, Bergen, Norway), following the constructor protocol. Each triplicate of culture was analyzed with technical duplicates.

Bacteria

Concentration and viability of free-living bacteria associated to *P. delicatissima* cultures were assessed using flow cytometry. To estimate bacteria viability, bacteria were analyzed after 15 min incubation with a final concentration of 1/10000 of the commercial solution of SYBR Green I (Molecular probes, Invitrogen, Eugene, Oregon, USA) and propidium iodide (PI, Sigma, St. Louis, MO, USA) at 10 µg ml^{-1}.

Bio-dilution

When cells divide, the cytoplasm and its content are divided between the two daughter cells. To assess the bio-dilution effect at steady state, the production rate (in arbitrary units per cell per day (AU $cell^{-1}$ d^{-1}) of lipids (BODIPY) and chlorophyll (FL3) can be calculated by multiplying the flow cytometric value (in AU $cell^{-1}$) by the acclimated specific growth rate (in d^{-1}).

Statistics

Effects of concentrations of Fe and Cu on the physiological parameters of *P. delicatissima* were tested using one way ANOVAs with the software StatGraphics Plus (Manugistics, Inc, Rockville, MD, USA). The test of rank used was the Tukey test (variance homogeneity was first tested and confirmed for all the parameters). Discriminant analysis was also performed to test the differences between the different cultures. For all statistical results, a probability of $p < 0.05$ was considered significant.

Acknowledgements

This work was supported by the ANR program (ICOP, ANR-10-JCJC-606), the French ministry of research (MENRT grant) to AL, Region Bretagne grant, SAD Volet 1, PSEUDOPE to HH and CNRS EC2CO Microbien to PS.

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6.3. Conclusion

Cette étude a permis de montrer que non seulement la limitation en fer et cuivre n'entraînait pas de production d'acide domoïque par une souche non toxique de *P. delicatissima*, mais également que les limitations en fer et cuivre n'induisaient pas les mêmes modifications physiologiques. Nous avons ainsi pu conclure que la carence en cuivre des cultures de *P. delicatissima* n'induisait pas une limitation en fer, signifiant que chez cette espèce la demande cellulaire en cuivre pour le transport du fer est minoritaire par rapport aux autres besoins en cuivre, ou que ces cellules possèdent un système d'acquisition du fer indépendant du cuivre. Cette conclusion, qui va à l'encontre d'une partie des théories actuelles (Wells et al., 2005; Maldonado et al., 2006), rejoint cependant celle de Annett et al. (2008), qui ont montré que pour six souches de diatomées sur huit étudiées (et cinq espèces sur six), le besoin en cuivre pour le transport haute affinité du fer est faible par rapport aux autres demandes cellulaires. Nous avons aussi pu montrer que les co-limitations fer-cuivre induisent surtout une limitation en fer des cultures, la limitation cuivre semblant secondaire et pouvant être compensée par les cellules par des modifications physiologiques pour maintenir leur taux de croissance. Par contre le cuivre devient indispensable quand le fer est limitant, ce qui laisse penser que chez cette espèce le fer et le cuivre peuvent tout de même être impliqués dans des mécanismes communs et/ou complémentaires (respiration, superoxyde dismutases contenant fer ou cuivre, ...).

La disponibilité en métaux influe donc la physiologie des *Pseudo-nitzschia*, qu'il y ait carence ou toxicité, et de fortes différences existent entre espèces. Il semble que la production d'acide domoïque de *P. multiseries* ne soit en revanche pas modifiée par l'ajout de cuivre et qu'une espèce non toxique soumise à un stress en fer ou cuivre (limitation ou toxicité) ne soit pas capable de produire de l'acide domoïque. Ces résultats n'invalident cependant pas l'hypothèse de Wells et al. (2005), qui reliaient la production d'acide domoïque au besoin cellulaire d'acquérir le cuivre pour transporter le fer. Au vu des données physiologiques, la demande cellulaire en cuivre pour le transport du fer chez *P. delicatissima* semble en effet être minoritaire, voire nulle, par rapport aux autres besoins en cuivre. Une autre hypothèse peut être postulée: si l'acide domoïque est effectivement utilisé pour acquérir le fer lié aux sidérophores excrétés par les bactéries (Wells et al., 2005), la production d'acide domoïque pourrait être régulée par la concentration en sidérophores dans le milieu. Le lien entre fer, cuivre, production d'acide domoïque et bactéries doit encore être approfondi.

SYNTHÈSE, CONCLUSIONS ET PERSPECTIVES

1. La physiologie des cellules

Le premier but de cette thèse était de suivre les modifications physiologiques des cellules de *Pseudo-nitzschia*, d'en déduire leur état physiologique et savoir si les cellules étaient stressées ou pas. En cas de stress, nous avons pu déterminer une partie des adaptations mises en place par les cellules pour contrecarrer et survivre à ce stress (Tableau 1). Pour savoir si la cellule est stressée ou pas, la mesure du taux de croissance uniquement ne suffit pas. Par exemple, lors de l'expérience de toxicité du cuivre (Lelong et al., 2012a - article 5), la croissance de *P. multiseries* n'est pas modifiée par l'exposition à 50 $\mu g\ l^{-1}$ de cuivre alors que les modifications physiologiques mettent en évidence un stress. Ce stress n'est juste pas assez important pour se répercuter sur la croissance, les cellules développant sûrement des mécanismes de compensation. Dans ce cas, il faut également différencier stress à court, moyen et long terme. Il est plus probable qu'à court terme, les cellules soient capables de maintenir leur taux de croissance (première génération de *Pseudo-nitzschia* soumise à une carence en fer (Bates et al., 2001), ou à l'inverse ne soient pas adaptées à ce stress et donc incapables de croître (*P. delicatissima* soumise à une dose toxique de cuivre, Lelong et al., 2012a - article 5). A moyen et long terme, les cellules peuvent ne plus être capables de maintenir leur taux de croissance (carences en fer et/ou cuivre, Lelong et al., in prep.-c - article 6) ou au contraire s'adapter et recouvrer leur taux de croissance (adaptation des cellules de *P. multiseries* à des carences en fer, (Wells et al., 2005). Ces travaux mettent donc en évidence l'importance de mesurer d'autres paramètres physiologiques et de ne pas se contenter de la croissance. De même, l'efficacité de photosynthèse, souvent utilisée comme proxy de l'état de santé des microalgues (par exemple McMinn et al., 2008) ne suffit pas. Par exemple, lors d'une limitation en cuivre (Lelong et al., in prep.-c - article 6), l'efficacité de photosynthèse des cellules de *P. delicatissima* n'est pas modifiée, alors que leur croissance, tout comme les autres paramètres physiologiques, l'est. Il est donc important de suivre différents paramètres physiologiques de façon simultanée pour mieux comprendre les mécanismes mis en place par les cellules pour survivre à un stress, quelque soit le phylum et la toxicité de l'espèce étudiée. Les différentes mesures que nous avons utilisées permettent non seulement de savoir si les cellules sont stressées mais également d'étudier de quelle façon le stress modifie la physiologie cellulaire (ou la cellule modifie sa physiologie en réponse au

stress) et d'en déduire une partie des adaptations physiologiques mises en place pour la survie cellulaire.

Tableau 1 : Tableau récapitulatif des modifications physiologiques observées chez les cellules en cas de modifications des facteurs biotiques ou abiotiques.

	Croissance	Mortalité	FL3	QY	Estérases	Lipides
Sans bactéries	+	→	→	→	-	-
Témoin	→	→	→	→	→	→
Compétition	→	→	+	?	+ ou ++	+ ou ++
Faible toxicité cuivre (*P. delicatissima*)	-	→	+	-	++	+ ou ++
Limitation cuivre (*P. delicatissima*)	-	→	++	→	+	++
Limitation fer (*P. delicatissima*)	-	→	-	-	+	+
Fortes limitations et co-limitations (*P. delicatissima*)	--	→	--	--	- ou --	→
Avec autres bactéries (*P. multiseries*)	--	++	--	-	+	→
Forte toxicité cuivre (*P. delicatissima*)	--	++	+	-	++ puis --	++

- ou -- = valeur < 50 % ou > 50 % en dessous du témoin
+ ou ++ = valeur < 50 % ou > 50 % au dessus du témoin

Combiner différentes mesures physiologiques nous a permis de mettre en évidence qu'un stress, quel qu'il soit, stimule l'activité des estérases (sauf en cas de fortes limitations en fer et/ou cuivre, les cellules ayant trop peu d'énergie). Cette stimulation est très probablement non spécifique puisque mise en jeu en cas de fortes doses de cuivre (Lelong et al., 2012a - article 5), d'une limitation intermédiaire en fer ou cuivre (Lelong et al., in prep.-c - article 6), ou encore en cas de compétition avec une autre diatomée (Lelong et al., in prep.-a - article 3). Elle semble intervenir aussi bien à court qu'à moyen et long terme. Par contre, pour que ce mécanisme se mette en place et se maintienne, il est nécessaire que les cellules aient assez d'énergie. Ainsi, les cellules de *P. delicatissima* soumises à une forte dose de cuivre pendant plusieurs jours meurent, la production d'estérases est donc stoppée (Lelong et al., 2012a -

article 5). De même, les cellules de *P. delicatissima* soumises à une forte carence en fer, ont une photosynthèse peu efficace, ne poussent pas et n'ont pas de lipides stockés ; elles n'ont donc pas assez d'énergie pour produire des estérases (Lelong et al., in prep.-c - article 6). Le stockage de lipides est lui aussi dépendant de l'état des cellules. Si toute l'énergie disponible pour les cellules n'est pas utilisée, celles-ci stockent cette énergie excédentaire sous forme de lipides, principalement quand elles sont stressées, aux dépens des glucides (Giordano et al., 2001). Mais pour que les estérases soient produites, ou les lipides stockés, les cellules doivent acquérir suffisamment d'énergie, par le biais de la photosynthèse suivie de la respiration. Lors de toutes les expériences menées dans cette thèse, tous les paramètres physiologiques mesurés peuvent être liés les uns aux autres et permettent ainsi de mieux comprendre l'état de stress des cellules, la façon dont les facteurs biotiques ou abiotiques modifiés perturbent la physiologie cellulaire et dont les cellules se protègent (Tableau 1). A la différence du milieu naturel, les cultures permettent de ne faire varier les conditions que d'un facteur. En milieu naturel, les espèces sont soumises à plusieurs stress simultanés, avec forcément des variations de plusieurs paramètres physiologiques, d'où l'intérêt de ces nombreuses mesures et des études en laboratoire en amont d'études de terrain.

Ces mesures ont en plus l'intérêt d'être potentiellement applicables à toutes les espèces de phytoplancton unicellulaire, sous condition qu'il puisse être analysé au cytomètre en flux. Cela permet donc de comparer la réaction à un même stress de différentes espèces, du même phylum ou de phylums différents (par exemple dinoflagellés et diatomées). Nous avons pu vérifier que cette approche fonctionne lors d'une collaboration avec Dianne Jolley. Toutes les mesures physiologiques ont été appliquées à des diatomées (*Pseudo-nitzschia* spp., *Chaetoceros neogracile* et *Phaeodactylum tricornatum*), un dinoflagellé (*Alexandrium minutum*) et deux algues vertes (*Tetraselmis* sp. et *Dunaliella tertiolecta*), exposés à des doses toxiques de cuivre. Cela nous a permis de constater que les réponses physiologiques varient en fonction de l'espèce concernée et peuvent même être totalement opposées. Appliquer ces mesures à différentes espèces peut donc permettre de mieux comprendre l'apparition de blooms, les successions d'espèces en milieu naturel, la réaction du phytoplancton en cas de stress, …

Pour compléter les mesures que nous avons mises en place, il serait intéressant de développer en parallèle des mesures de photosynthèse et de respiration, des mesures de l'énergie directement disponible pour les cellules (par exemple mesurer l'ATP intracellulaire).

De plus, les mesures de lipides, grâce à la sonde BODIPY, permettent de donner une idée globale de la quantité de lipides intracellulaires, mais pas de déterminer la nature ni la répartition de ces lipides. Des expériences sont d'ailleurs en cours pour déterminer les différentes classes de lipides de *P. delicatissima*, et mieux comprendre l'impact de certains facteurs biotiques et abiotiques sur la composition lipidique de *P. delicatissima* (des dosages sont actuellement en cours sur une expérience de carence en cuivre). Pour être plus précis sur les réponses physiologiques à un stress particulier, il faudrait ensuite pouvoir doser des protéines spécifiques, tester l'activité d'enzymes connues et ciblées en fonction des différents facteurs de stress testés ou encore évaluer l'expression de gènes codant pour certaines protéines plus ou moins spécifiques dans la réponse au stress et si possible trouver les gènes responsables de la production d'acide domoïque (le génome de *Pseudo-nitzschia* est en cours de séquençage). A plus long terme, il serait intéressant de faire des études dans un premier temps en mésocosmes (si possible placés en milieu naturel, e.g. des bouteilles fixées à une bouée dans l'océan), pour vérifier que les espèces subissent des modifications physiologiques qui peuvent être mesurées, pour ensuite faire des études avec plusieurs espèces avant de passer aux mesures en milieu naturel (suivi temporel, effet de fertilisations, …).

2. Lien entre physiologie et production d'acide domoïque

Dans la majorité des études réalisées sur *Pseudo-nitzschia* (par exemple Bates et al., 2001, Wells et al., 2005, Fehling et al., 2005), seules la croissance et l'acide domoïque sont mesurées. Pourtant, d'après nos résultats, il n'y a pas de lien direct entre ces deux paramètres (Figure 1). L'intérêt de notre étude est de mesurer d'autres paramètres physiologiques. En effet, le suivi de la physiologie a permis de suivre la photosynthèse et le métabolisme primaire (avec l'activité des estérases), ainsi que le stockage d'énergie excédentaire (entre autres avec le contenu lipidique et l'acide domoïque). L'acide domoïque étant un métabolite secondaire, sa production est directement liée à l'état physiologique de la cellule. Nous avons ainsi pu observer que la production d'acide domoïque est souvent précédée d'une augmentation de l'activité des estérases (i.e. du métabolisme primaire). Nous avons également observé que l'activité de ces estérases était augmentée en cas de stress (Lelong et al., 2012a, Lelong et al., in prep.-c - articles 5 et 6). Les estérases étant des enzymes non spécifiques, ces deux mécanismes ne sont peut être pas directement liés. Deux hypothèses peuvent donc être posées: (i) la production d'acide domoïque nécessite de nombreuses enzymes, le métabolisme

primaire, et plus précisément la production d'enzymes, dont les estérases, est donc augmenté et/ou (ii) les cellules sont soumises à un stress qui induit l'augmentation de l'activité des estérases et la production d'acide domoïque, deux mécanismes indépendants. *A priori*, aucun lien direct n'a pu être établi entre la production d'acide domoïque et la quantité de lipides stockés, ceux-ci étant stockés quand les cellules ont trop d'énergie ou sont stressées. La production d'acide domoïque peut se faire (i) quand il n'y a pas de lipides de réserve (*P. multiseries* mise en culture avec les bactéries associées à *P. delicatissima*, Lelong et al., in prep.-b - article 4), l'énergie est ainsi directement utilisée et n'est pas stockée ou (ii) quand il y a des lipides de réserve (*P. multiseries* soumise à des doses toxiques de cuivre, Lelong et al., 2012a - article 5). Quand les cellules ne produisent pas assez d'énergie, l'énergie stockée sous forme de lipides peut alors être utilisée par les cellules pour la production d'acide domoïque. A l'inverse, chez les cellules ayant plus d'énergie que nécessaire pour la production d'acide domoïque, le stockage de lipides augmente. Tous les paramètres que nous avons mesurés permettent en partie de comprendre la répartition énergétique au sein des cellules, et surtout de mieux identifier son utilisation, par exemple pour la synthèse d'acide domoïque. Toutes ces mesures semblent nécessaires pour mieux comprendre quand et pourquoi, la production de toxine varie. D'autres mesures pourraient être effectuées, pour compléter le schéma de production de l'acide domoïque. Le dosage des glucides en complément de celui des lipides permettrait d'avoir une meilleure idée de la localisation de l'énergie stockée, tandis que le dosage des différentes classes de lipides permettrait de savoir si ce sont bien les lipides de stockage dont la quantité augmente, ou plutôt les lipides de structure. Là encore, les mesures sont faites en conditions contrôlées, en milieu naturel, les espèces se divisent rarement à leur taux maximal. Le passage par des mesures en laboratoire est obligatoire pour pouvoir détailler les variations physiologiques en réponse à un seul facteur, chose impossible en milieu naturel.

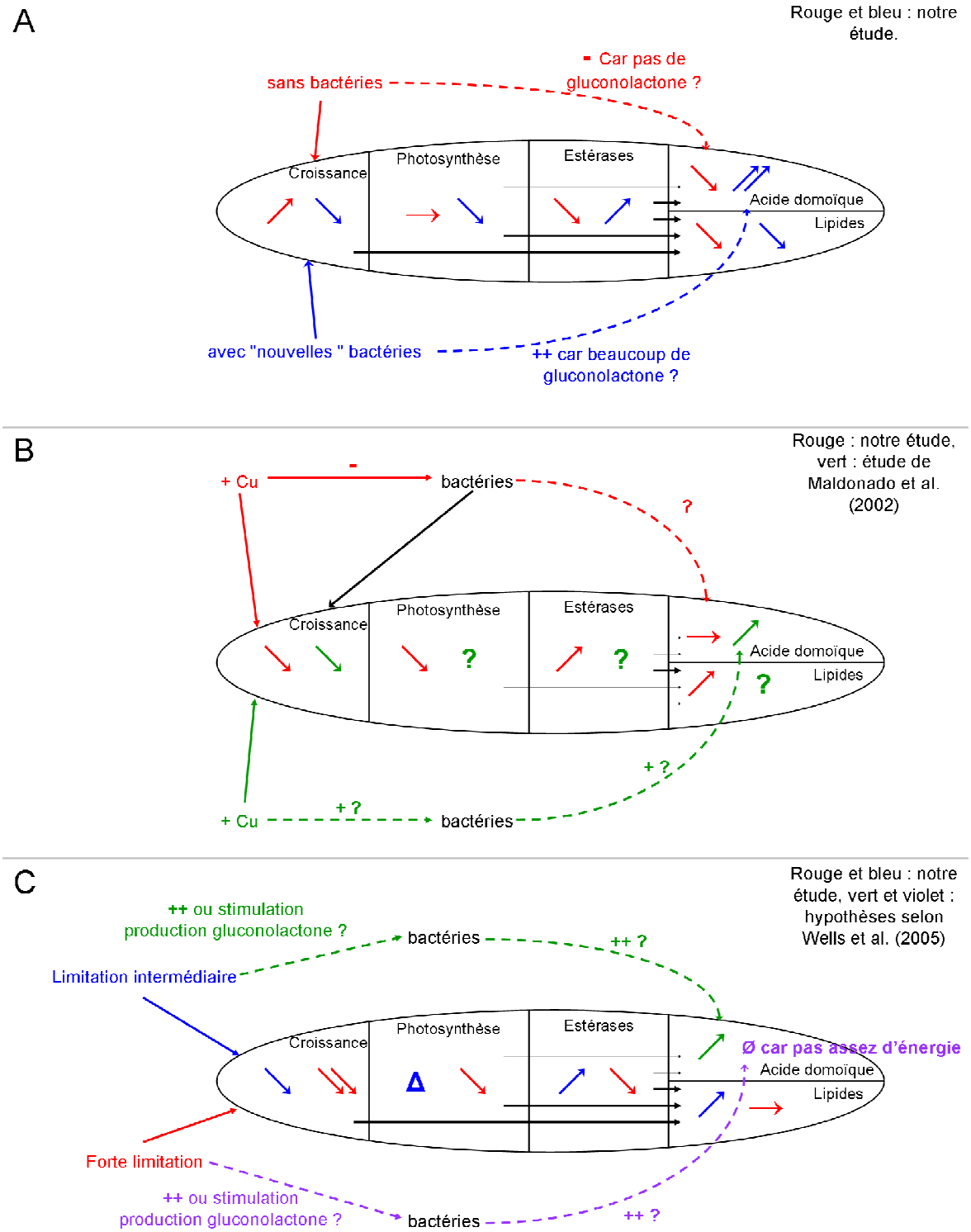

Figure 1. A. Scénario de modifications physiologiques et production d'acide domoïque suivant la présence ou l'absence de bactéries, par rapport à un témoin non axénique, chez *P. multiseries*. B. Scénario de modifications physiologiques et de production d'acide domoïque en cas de forte concentration de cuivre dans l'eau. C. Scénario de modifications physiologiques et de production d'acide domoïque en cas de limitations en fer et/ou cuivre. En noir, les relations démontrées lors de cette thèse, en traits pleins, nos résultats, en traits pointillés, les hypothèses posées pour expliquer les résultats de Maldonado et al. (2002) dans le scénario B ou les résultats de Wells et al. (2005) dans le scénario C.

3. La modification de la production d'acide domoïque

La présence de bactéries est connue pour être indispensable à la production d'acide domoïque (Bates et al., 1995, Douglas et al., 1993, Kaczmarska et al., 2005). Nous avons cependant pu démontrer qu'une réduction de 90 % de la concentration bactérienne au moment de l'inoculum était suffisante pour stopper la production d'acide domoïque (Figure 1A, Lelong et al., in prep.-b - article 4). De plus, cette production reste basse même lorsque la concentration bactérienne de la culture "axénique" rejoint celle de la culture non axénique (Lelong et al., in prep.-b - article 4). Cela peut venir du fait que seules certaines bactéries sont capables d'induire la production d'acide domoïque et que ces bactéries ont été plus efficacement éliminées par les antibiotiques. En effet, l'hypothèse actuelle voudrait que la production d'acide domoïque soit induite par la présence de gluconolactone/acide gluconique, un chélatant des métaux produit uniquement par certaines bactéries (Osada and Stewart, 1997, Stewart, 2008, Stewart et al., 1997). Ainsi, seulement une partie des bactéries associées à *P. multiseries* produirait ce chélatant et serait éliminée par le traitement antibiotique. Les bactéries associées à *P. delicatissima* entraînant une très forte production d'acide domoïque, nous pouvons supposer que ces bactéries produisent beaucoup de gluconolactone/acide gluconique. Il serait intéressant pour les expériences futures, de doser cet acide gluconique, en parallèle du dosage de l'acide domoïque.

Lorsque nous avons exposé *P. delicatissima* à des fortes doses de cuivre avec ajout d'acide domoïque, nous avons pu noter que l'acide domoïque ne protégeait pas les cellules de *Pseudo-nitzschia* de l'effet toxique du cuivre (Lelong et al., 2012a - article 5). Par contre l'acide domoïque favorisait la croissance bactérienne, et ce indépendamment de la présence de cuivre. Ce résultat suggère que les bactéries pourraient tirer profit de l'acide domoïque et l'utiliser pour leur propre métabolisme (acquisition de métaux ?), et non pour se protéger du cuivre. De même, lors de l'expérience exposant *P. multiseries* à de fortes doses de cuivre, la production d'acide domoïque n'était pas modifiée, tandis que les bactéries étaient tuées. Pourtant, Maldonado et al. (2002) ont observé une augmentation de la production d'acide domoïque lors d'une exposition au cuivre (dose médiane à celles que nous avons utilisées). Cela pourrait s'expliquer par une augmentation de la population bactérienne lors de leur étude (Figure 1B). En effet, certaines bactéries sont stimulées par la présence de cuivre (par exemple cas des bactéries associées à *D. tertiolecta*, données personnelles), la croissance de

ces bactéries pouvant entraîner une stimulation de la production d'acide domoïque. Dans ce cas, il reste à déterminer si toutes les bactéries sont capables d'utiliser l'acide domoïque et l'utilisation qu'elles peuvent en faire. Les bactéries associées à *P. delicatissima* se multiplient avec une algue non-toxique, et ne sont donc *a priori* pas adaptées à la présence d'acide domoïque. Elles ont pourtant su utiliser l'acide domoïque ajouté au milieu (Lelong et al., 2012a - article 5). Si les bactéries utilisent en premier l'acide domoïque, quel peut donc être l'intérêt pour *P. multiseries*, et toutes les autres espèces toxiques, à en produire ? En effet, du "point de vue" de *Pseudo-nitzschia*, produire de l'acide domoïque semble se faire au détriment de la croissance, qui peut même être nulle (Lelong et al., 2012a - article 5). Ainsi, dans la littérature, des espèces/souches toxiques se multiplient moins vite en moyenne que les espèces/souches non toxiques, même cultivées dans des conditions identiques (Thessen et al., 2009). L'acide domoïque n'a pas de pouvoir allélopathique (Lundholm et al., 2005), pas plus qu'il ne semble rendre *P. multiseries* plus compétitive (Lelong et al., in prep.-a - article 3). Il ne limite pas le broutage (Bargu et al., 2003, Thessen et al., 2010) et n'a *a priori* pas d'effets négatifs majeurs sur les filtreurs (Jones et al., 1995, Douglas et al., 1997), seulement sur les derniers maillons trophiques, qui n'ont aucun contact direct avec *Pseudo-nitzschia*. Le rôle écologique de l'acide domoïque est donc inconnu et semble relativement mineur, même si son impact sur la santé humaine et l'économie est lui très important. D'autant plus que les espèces/souches non toxiques sont également retrouvées partout dans le monde (Lelong et al., 2012b - article 1) et ont donc probablement mis en place un autre mécanisme, plus efficace, pour acquérir les métaux traces. Ainsi, quelque soit la communauté bactérienne associée à *P. delicatissima* (Lelong et al., in prep.-b - article 4), les algues ont le même taux de croissance et une physiologie quasiment similaire. L'hypothèse que *P. delicatissima* produise un autre chélatant, non utilisé par les bactéries et donc plus efficace pour les cellules de *Pseudo-nitzschia*, est à envisager. Cela pourrait également expliquer la plus forte croissance des espèces/souches non toxiques.

Une observation plus détaillée des communautés bactériennes, permet de mettre en évidence des différences suivant les espèces de *Pseudo-nitzschia* (Annexe 1). Dans cette thèse, seuls les paramètres "morphologiques" (FSC et SSC) et la mortalité des bactéries ont été suivis. Par exemple, en cas de fortes doses de cuivre, les bactéries peuvent être sensibles et mourir (cas des bactéries associées à *P. multiseries*, (Lelong et al., 2012a - article 5), ne pas être impactées (cas des bactéries associées à *P. delicatissima*, (Lelong et al., 2012a - article 5) ou encore être stimulées (cas des bactéries associées à *D. tertiolecta*, données personnelles).

La communauté peut également être modifiée au cours de la croissance de l'algue (deux semaines, Annexe 1) ou au cours du temps (Sapp et al., 2007). Ainsi, le nombre de bactéries associées à *P. delicatissima* a fortement diminué entre le moment de son isolement et aujourd'hui, avec une moyenne supérieure à 30 millions de bactéries par ml en novembre 2009, pour une moyenne de 5 millions en janvier 2011 (au moment de la fin de phase exponentielle de *P. delicatissima*). Les cultures semblent s'adapter à "leur" communauté bactérienne, comme le montre l'apparition d'une phase stationnaire chez *P. delicatissima* après plus d'un an de culture (données personnelles). L'adaptation à une communauté bactérienne au cours des repiquages pourrait expliquer la perte de toxicité observée avec les années chez *P. multiseries* and *P. australis* (Bates, 1998, Rhodes et al., 2004). Elle pourrait également être liée à la diminution de taille observée chez les diatomées avec le temps et n'être pas directement liée à l'adaptation aux communautés bactériennes. Le nombre de bactéries, ainsi que le ratio bactéries/*Pseudo-nitzschia*, changeant au cours de la croissance de l'algue, pourraient expliquer pourquoi certaines espèces produisent de l'acide domoïque en phase exponentielle (Lelong et al., 2011, Adams et al., 2000, Garrison et al., 1992, Pan et al., 2001) et d'autres en phase stationnaire (Fehling et al., 2004, Thessen et al., 2009, Trainer et al., 2009). Malheureusement, seule notre étude (Lelong et al., 2012b - article 1) a suivi la croissance bactérienne. La production d'acide domoïque était corrélée dans cette étude au ratio bactéries/*Pseudo-nitzschia*. Une production d'acide domoïque en phase stationnaire serait donc due à une croissance bactérienne qui continue quand les *Pseudo-nitzschia* sont en phase stationnaire, entraînant une augmentation du ratio bactéries/*Pseudo-nitzschia*.

De même, lors de carences en fer et/ou cuivre sur *P. delicatissima*, la communauté bactérienne est affectée (Annexe 1). Si on part du principe que les bactéries induisent la production d'acide domoïque pour leur propre acquisition de métaux (ou que l'excrétion de gluconolactone/acide gluconique induit la production d'acide domoïque), nous pouvons supposer qu'en cas de limitation cuivre et/ou fer, les bactéries vont soit induire une augmentation de la production d'acide domoïque, soit produire plus de gluconolactone/acide gluconique qui va induire une augmentation de la production d'acide domoïque (Figure 1C). Dans les deux cas, une souche de *Pseudo-nitzschia* toxique produira plus d'acide domoïque en cas de limitation. Cela a été prouvé par Wells et al. (2005) qui ont montré que *P. australis* produit plus d'acide domoïque en cas de limitation en cuivre ou en fer et en cuivre alors que *P. multiseries* produit plus d'acide domoïque en cas de limitation fer et/ou cuivre. Par contre, ils ont également observé une diminution de la production d'acide domoïque après adaptation

des cultures au milieu limitant en fer. Cela peut s'expliquer de différentes manières : (i) les algues ont mis en place d'autres mécanismes, sont ainsi adaptées à la carence et n'ont plus besoin de produire autant d'acide domoïque ou (ii) les bactéries sont adaptées à la carence et produisent moins de gluconolactone/acide gluconique ce qui induit une plus faible production d'acide domoïque ou encore (iii) les bactéries sont adaptées à la carence et induisent moins d'acide domoïque car elles ont réduit leur métabolisme et ont moins besoin de fer/cuivre. Pour mieux comprendre l'effet d'autres limitations (par exemple en phosphate, silice, …), il conviendrait donc de refaire les expériences en suivant *a minima* la concentration bactérienne et si possible leur "morphologie" et physiologie (i.e. leur survie, …).

Si le déclencheur réel de la production d'acide domoïque reste inconnu, il semble que le rôle des bactéries soit incontournable et que la présence (en tout cas de certaines d'entre elles) soit indispensable. La question majeure reste de déterminer pourquoi et comment les bactéries induisent cette production d'acide domoïque. Est-ce une induction directe ? Ou la compétition avec les bactéries induit-elle la synthèse d'acide domoïque, dont les bactéries ont su détourner le rôle à leur propre profit ? Pourquoi la compétition avec une autre diatomée n'induit pas la production d'acide domoïque ? Pourquoi des variations en nutriments peuvent modifier la production d'acide domoïque (par exemple la limitation en phosphore augmente la production d'acide domoïque, Fehling et al., 2004) ? Nous avons pu voir dans nos expériences que le stress seul n'induit pas la production d'acide domoïque. Tout est-il donc lié aux bactéries et à leur concentration ? Si la production d'acide domoïque semble avoir des effets négatifs sur les cellules, quels mécanismes ont été mis en place par les cellules non toxiques pour "remplacer" l'acide domoïque ? Puisque seules certaines bactéries semblent capables d'induire la production d'acide domoïque et à des niveaux variables, il semble également intéressant de déterminer la diversité bactérienne associée aux différentes souches d'algues et d'aller plus loin dans la détermination d'espèces bactériennes, et/ou au moins de déterminer quelles bactéries sont inductrices de la production d'acide domoïque et lesquelles ne le sont pas.

Nous n'avons bien entendu pas testé tous les facteurs et il est possible que l'acide domoïque ait un rôle positif pour les espèces de *Pseudo-nitzschia* dans certaines conditions. Cette production se faisant au détriment de la croissance, il faut que cette production soit la seule possibilité pour les algues de survivre. La présence de bactéries augmentant la

production d'acide domoïque pourrait être une "erreur". Les bactéries pourraient induire la production d'acide domoïque (directement ou indirectement) alors que la cellule n'en a pas besoin, entraînant un avantage compétitif pour ces bactéries. En milieu naturel, cet avantage des bactéries sur les espèces toxiques pourrait expliquer la disparition récente d'évènements toxiques à *P. multiseries* dans le monde (Lelong et al., 2012b). Bien entendu, ces expériences doivent être refaites sur d'autres espèces, pour confirmer que la production d'acide domoïque se fait au détriment de la croissance, et dans des conditions se rapprochant le plus possible du milieu naturel, avant de passer à des mesures (et plutôt des suivis) en milieu naturel.

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ANNEXES

Annexe 1 – Cytogrammes des communautés bactériennes

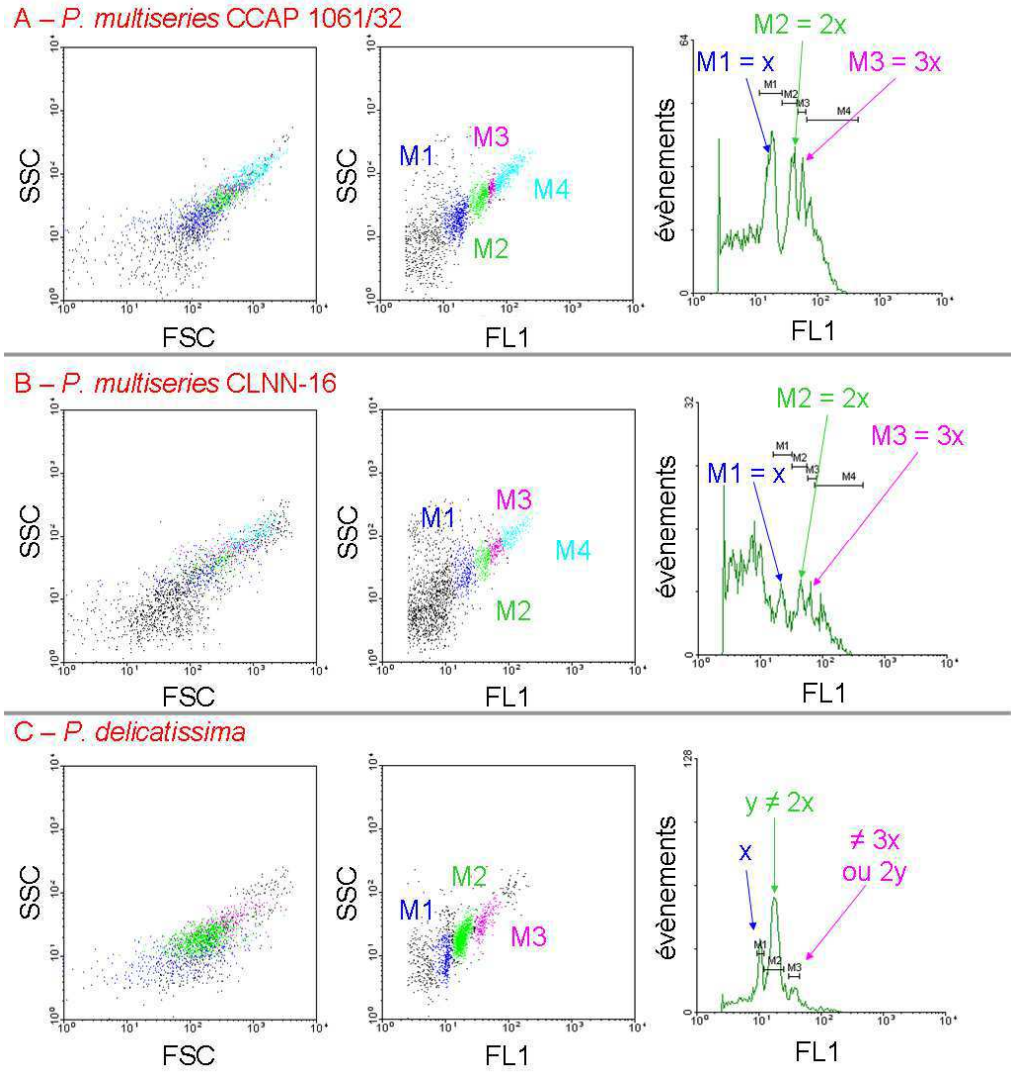

Figure 1. Cytogrammes des populations bactériennes de *P. multiseries* CCAP 1061/32 (A), *P. multiseries* CLNN-16 (B) et *P. delicatissima* (C) au cours des expériences de suivi physiologique (Lelong et al., in press.-b - article 2), compétition (cellules de *Pseudo-nitzschia* seules, Lelong et al., in prep.-a - article 4) et échange de communauté bactérienne (*P. delicatissima* avec ses propres bactéries, Lelong et al., in prep.-b - article 3) respectivement. Chaque couleur représente une population bactérienne, avec un contenu en ADN différent.

Les figures 1 à 3 sont des cytogrammes des communautés bactériennes associées à *P. multiseries* ou *P. delicatissima*. Pour pouvoir les détecter au cytomètre en flux, les bactéries ont été marquées au SYBR Green. Les cytogrammes représentent les paramètres "morphologiques" des communautés bactériennes (FSC, diffusion de la lumière aux petits angles et SSC, diffusion de la lumière aux grands angles) où la fluorescence FL1 des bactéries due au marquage SYBR Green. Sur la figure 1, on peut voir que la population bactérienne est

différente non seulement entre espèces mais également entre souches différentes d'une même espèce. Les populations bactériennes ont différents paramètres morphologiques mais également un contenu en ADN différent. Sur la souche CCAP 1061/32 (Figure 1A), on peut voir différents pics sur l'histogramme FL1, qui correspondent à différents contenu en ADN. Chaque pic ayant une fluorescence multiple du premier pic, il s'agit fort probablement d'amas d'une, deux bactéries ou plus, bactéries associées à même type (i.e. même contenu en ADN) (cf. Lelong et al., in press - article 2). Ces amas sont visibles dans la culture de *P. multiseries* CLNN-16, de façon un peu moins nette, avec beaucoup plus de bactéries ou débris avec une très faible fluorescence verte. Chez *P. delicatissima*, les différentes pics en FL1 ne sont pas des multiples et représentent donc plus probablement différents types de bactéries, avec un contenu en ADN différent.

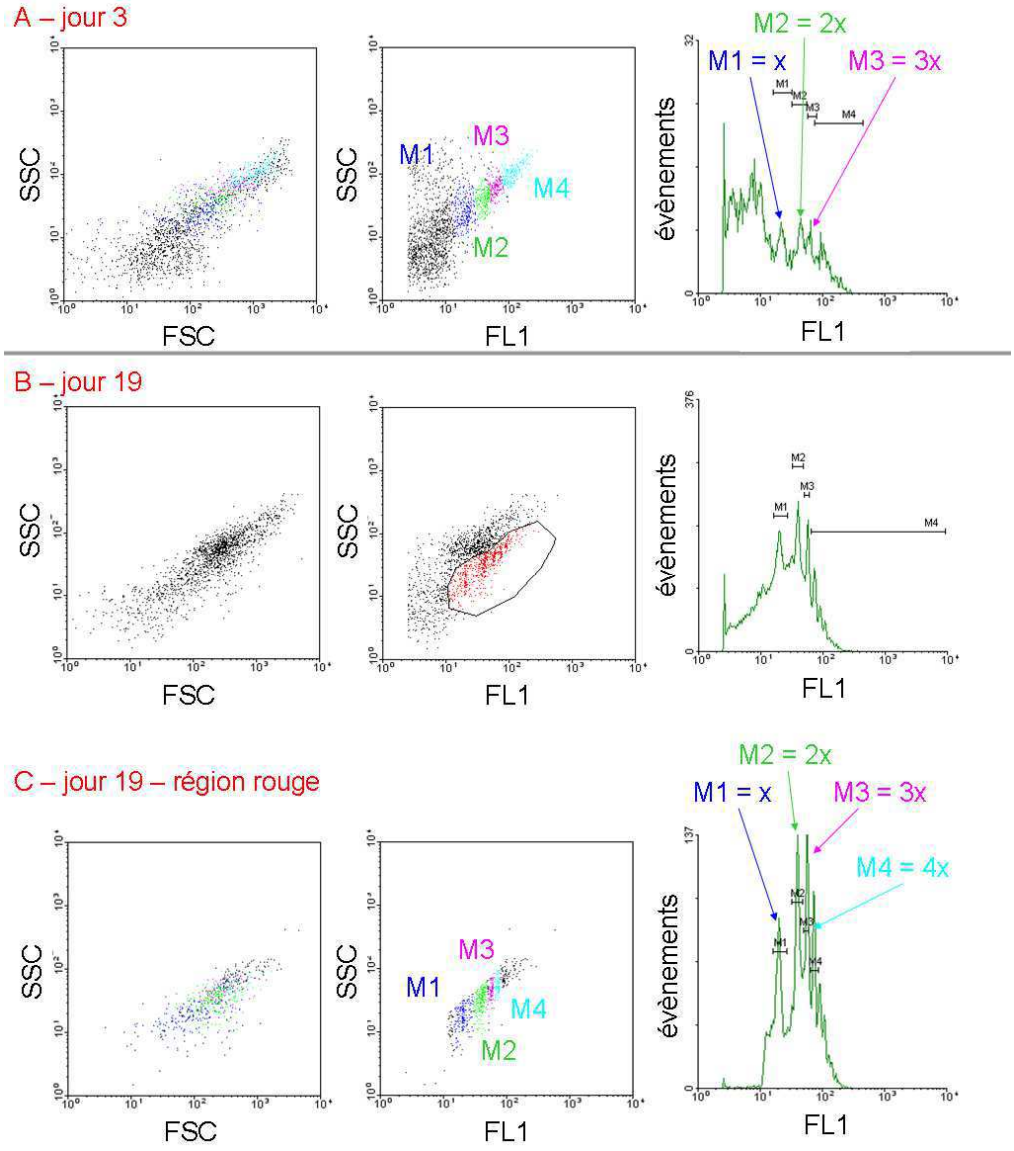

Figure 2. Cytogrammes des populations bactériennes de *P. multiseries* CLNN-16 au cours de l'expérience de compétition (cellules de *Pseudo-nitzschia* seules, Lelong et al., in prep.-a - article 4) après 3 jours (A) et 19 jours (B) de croissance.

La figure 2 correspond à la communauté bactérienne associée à *P. multiseries* CLLNN-16 au cours la même expérience de suivi de croissance (Lelong et al., in prep.-a - article 4), après 3 et 19 jours de croissance (Figure 2A et 2B respectivement). On peut voir qu'au cours de la croissance algale, une population apparaît (en noir, Figure 2B), population qui n'a pas de pics de FL1 visibles. Cette population avec une forte valeur de SSC pourrait correspondre à des agrégats bactériens de grande taille, sauf que leur fluorescence verte n'augmente pas. Par contre, quand on retire cette population, on voit apparaître les pics avec des valeurs multiples de fluorescence, qui montrent des agrégats. Le nombre d'agrégats semble augmenter avec le temps.

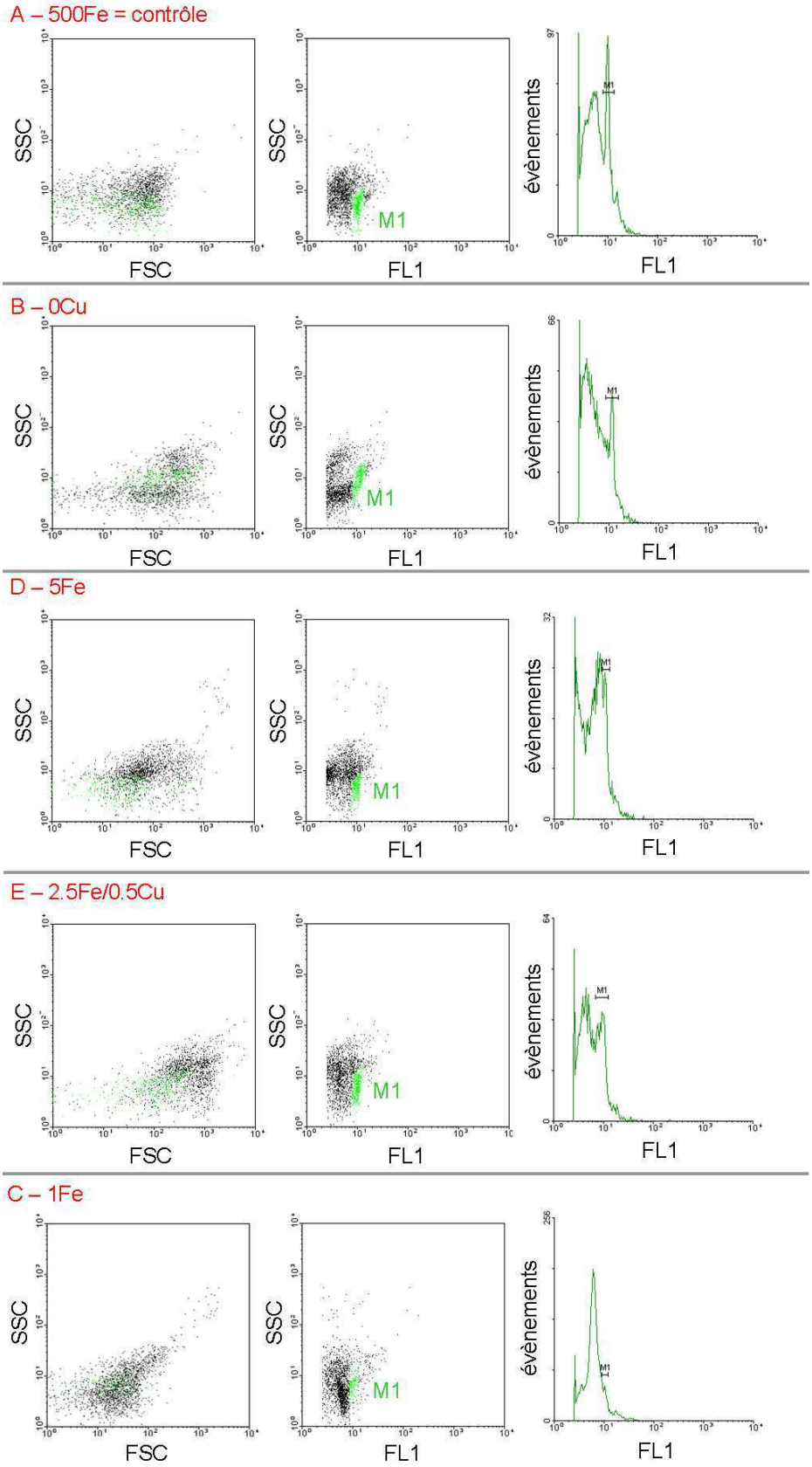

Figure 3. Cytogrammes des populations bactériennes de *P. delicatissima* au cours de l'expériences de limitation en métaux (Lelong et al., in prep.-d - article 6). A. culture témoin donc non limitée (500 nM total de fer et 20 nM total de cuivre). B. culture limitée strictement en cuivre (0 nM de cuivre). C. culture fortement limitée en fer (1nM total de fer). D. culture limitée en fer (5nM total de fer). E. culture limitée en fer et en cuivre (2.5 nM total de fer et 0.5 nM total de cuivre).

La figure 3 représente les communautés bactériennes associées à *P. delicatissima* maintenues en milieu AQUIL et après adaptation à des conditions limitantes en fer et/ou cuivre. On peut ainsi voir que les algues ne sont pas les seules à s'être adaptées. Quand on regarde par exemple le pic M1, plus le milieu est limitant, plus ce pic est petit comparé au nombre total de bactéries. Cette population bactérienne est donc probablement limitée elle aussi. Il est par contre difficile de déterminer si certaines espèces ont été favorisées ou s'il s'agit d'adaptations morphologiques et physiologiques des espèces, qui entraînent une modification des paramètres FSC, SSC et FL1, sans pour autant changer le nombre et le rapport des espèces.

Annexe 2 - Article 7 – A new insight into allelopathic effects of Alexandrium minutum *on photosynthesis and respiration of the diatom* Chaetoceros neogracile *revealed by photosynthetic-performance analysis and flow cytometry*

Microb Ecol
DOI 10.1007/s00248-011-9889-5

MICROBIOLOGY OF AQUATIC SYSTEMS

A New Insight into Allelopathic Effects of *Alexandrium minutum* on Photosynthesis and Respiration of the Diatom *Chaetoceros neogracile* Revealed by Photosynthetic-performance Analysis and Flow Cytometry

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Received: 16 December 2010 / Accepted: 6 June 2011
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Abstract The allelopathic effects of *Alexandrium minutum*, a toxic dinoflagellate, on the diatom *Chaetoceros neogracile* were evaluated using unialgal cultures evaluated by flow cytometry (FCM) and photosynthetic-performance analysis. Using FCM, we demonstrated that red chlorophyll fluorescence, relative cell size (Forward scatter of blue laser light, FSC) and cell complexity (Side scatter, 90°-angle scatter of blue laser light, SSC) significantly and rapidly decreased in *C. neogracile* cells exposed to *A. minutum*. Cells of *C. neogracile* exposed to *A. minutum* had fewer active photosynthetic reaction centers and sharply decreased photosynthetic efficiency. These effects were intensified with advancing *A. minutum* batch culture age and cell density. The supernatant of *A. minutum* contained the majority of the putative allelopathic compounds, and the biological activity of these compounds remained active less than 9 h after release. This paper describes for the first time specific effects of allelochemicals produced by *A. minutum* on the photosynthetic apparatus of microalgal target cells. The biochemical composition of *A. minutum* allelopathic agents, however, remains unknown and still needs to be investigated.

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Published online: 05 July 2011

Introduction

Among unicellular microalgal classes, Dinophyceae is one of the most heterogeneous groups. Some species are obligate autotrophs, such as *Gambierdiscus toxicus* [20], others are mixotrophic, such as *Heterocapsa triquetra* [21, 26], and others are heterotrophic, such as *Protoperidinium vorax* [39]. Within this group, many species are known to be toxic, including *Alexandrium minutum* [12], which is responsible for "red tide" blooms in some coastal waters [17], as in Brazil [31], Australia [18], or the Mediterranean Sea [14, 25, 51]. The genus *Alexandrium* can be found worldwide [7, 50, 51] and can be toxic to humans [27] through the production of paralytic shellfish toxins (PSTs), mainly saxitoxin and gonyautoxins. Within the genus *Alexandrium*, allelopathic, bio-active effects have been studied for almost 25 years [33]. Several species of the genus appear to have harmful effects upon ciliates [19], autotrophic protists [4, 47, 53, 57], and heterotrophic protists [46]. These effects differ from one target species to another [36, 43, 46, 47] and also depend upon *Alexandrium* species [4, 46, 48] and clones [19, 49]. Bacteria do not seem to be affected by putative allelopathic compounds [11]. Observed effects of *Alexandrium* species upon "target" species mostly are behavioral [19, 46] or involve cell lysis [4, 9, 19, 33, 46], but mechanisms mediating these responses are poorly understood. Research on cyanobacterial allelochemicals has revealed effects upon photosynthesis of the target cells, specifically the PSII-mediated, photosynthetic electron transport [13, 38, 40]. There are no reports, however of similar microalgal responses to dinoflagellates.

Allelopathy is characterized by the release of secondary metabolites into the medium that have beneficial or harmful

effects on target organisms [37]. Microalgal allelopathy has been known for at least 70 years [34]. Allelopathy may help species to outcompete others for nutrients [11, 46]. This hypothesis is strengthened by the observation that nutrient limitation can increase the allelopathic activity of cyanobacteria [52], freshwater dinoflagellates [36], or prymnesiophyte flagellates [45]. Allelopathy may also confer protection from grazers, as in *Prymnesium parvum* that kills its predator *Oxyrrhis marina* and subsequently eats it [45]. Fistarol and co-workers [11] showed that *Alexandrium* spp. can affect phytoplanktonic community structure at different trophic levels by modifying growth rates of species. Inhibiting growth or killing competitive species leaves more resources and space for the allelopathic species to bloom.

In the oceans, *A. minutum* co-occurs with many different species, including diatoms, such as *Chaetoceros* spp. which can be present after *A. minutum* blooms (réseau de surveillance du phytoplancton et des phycotoxines, REPHY, Ifremer, France). Cells of *A. minutum* and *Chaetoceros* spp. are found in the same areas (REPHY), sometimes at the same place and time, which means that there are processes mediating interactions and succession. In addition to the competition between these taxa, there seem to be allelopathic interactions [4, 11]. To elucidate interactions between *A. minutum* and *C. neogracile*, especially the effects of *A. minutum* upon *C. neogracile*, we focused the present study on the physiological status of both species when interacting.

Flow cytometry (FCM) is an established biomedical technology that was first adapted to measure microalgal physiology during the 1980s [6, 8, 56]. FCM allows rapid analysis of specific characteristics of individual cells in a population [22], such as morphological characteristics (inferred from light diffraction of the flow cytometer laser) and cellular chlorophyll content. FCM allows rapid analyses at the individual cell level, in contrast to whole-population measurements accomplished by spectrophotometric or bulk fluorometric methods. FCM also has been used to assess microalgal cyst and cell viability [6], dinoflagellate encystment [16], phytoplankton enzymatic activity [35], cell cycle [29] or other physiological analyses [22] using fluorescent probes.

Interactions between laboratory cultures of the toxic dinoflagellate *A. minutum* and the diatom *C. neogracile* were studied to assess allelopathic activity of *A. minutum*. Effect of age and cell density of *A. minutum* cultures was investigated. Finally, specific effects of supernatant from *A. minutum* cultures upon *C. neogracile* were explored to better understand how *A. minutum* allelochemicals may impact *C. neogracile* cells. FCM cell counts and measures of morphology and physiology, in terms of chlorophyll *a* fluorescence, were performed on *C. neogracile* cells. In addition, photosynthetic capacity of *C. neogracile* was analyzed using pulse amplitude-modulated fluorescence (PAM), and oxygen measurements. Photosystem II performance was measured using the OJIP transient curve, method applied recently to microalgae [1, 42]. The OJIP transient curve is a high resolution (<0.1 ms) kinetic representation of chlorophyll fluorescence under intense light exposure [2]. The transfer of energy along the photosynthetic apparatus can be monitored, with the opening and closing of the reaction centers (RC) of the cells and activation of the sequential intermediates, e.g. plastoquinone, visible in O, J, I and P steps.

Methods

Cell Culture

The strains AM89BM (isolated in 1989 in Brittany, France) of *A. minutum* Halim (1960) and CCAP 1010/3 of *Chaetoceros neogracile* Van Landingham (1968) were used in these experiments. Cultures were grown in filter and autoclaved L1 medium [15], at 16°C, with a dark:light period of 12:12 h (100 µmol photons m^{-2} s^{-1}). Cultures were not free of bacteria and grown without antibiotics. Each week, cultures were inoculated in triplicate. Before each sampling, cultures were mixed by gentle, manual shaking.

Experiments on Allelopathic Interactions

Microalgal interaction experiments were performed in 12-ml, pre-sterilized tubes (Deltalab eurotubo®) maintained in culture conditions with gentle agitation, in triplicate. Supernatant from *A. minutum* culture was separated by centrifugation (10 min, 800×*g*, 18°C) and filtered to 0.2 µm (acetate cellulose filters, Minisart, Sartorius, Göttingen, Germany) to eliminate bacteria. The supernatant was always prepared just before the experiment and added directly after filtration to *C. neogracile* cultures. The *A. minutum* cell pellet was resuspended in new L1 medium to obtain cells without supernatant. Un-inoculated L1 medium was used as a control. Cultures of *C. neogracile* used were always in exponential growth phase; whereas *A. minutum* cultures were always in stationary phase, with cell density ~65,000 cells ml^{-1} (except for Experiment 4).

Experiment 1—Effect of A. minutum Culture Fractions upon C. neogracile

Cells of *C. neogracile* were exposed to cultures, cells (without supernatant), and supernatant only of *A. minutum* (at a *C. neogracile: A. minutum* ratio of 60:1, with 5,000 cells ml^{-1} of *A. minutum*). Experimental tubes were sampled every 30 min for 3 h followed by FCM analyses.

Experiment 2—Long-term Effect of A. minutum upon C. neogracile

Supernatant of *A. minutum* (18-day cultures) was heated for 20 min at 100°C. Culture, supernatant, or heated supernatant of *A. minutum* cultures was added to *C. neogracile* cultures (at a *C. neogracile:A. minutum* ratio of 14:1). FCM analyses were performed after 3, 24, 48 and 96 h.

Experiment 3—Effect of A. minutum Cell Density upon C. neogracile

To test the effect of cell density of *A. minutum* upon *C. neogracile*, *C. neogracile* cultures were exposed to *A. minutum* cultures at experimentally-varied cell densities from 50 to 5,000 cells ml^{-1} (dilution in L1 medium, *C. neogracile: A. minutum* ratio between 4,000:1 and 40:1) to find the lowest density of *A. minutum* exhibiting an allelopathic effect upon *C. neogracile*. FCM analyses were performed after 4 h.

Experiment 4—Effect of A. minutum Culture Age upon C. neogracile

To test the effect of the age of *A. minutum* cultures upon *C. neogracile*, cultures of *A. minutum* of 6, 11, 19 and 27 days post-inoculation (diluted in L1 medium to obtain the same density of 7,000 cells ml^{-1}) were mixed with cultures of *C. neogracile* (*C. neogracile:A. minutum* ratio of 57:1, *A. minutum* cell density of 7,500 cells ml^{-1}). Cultures of *A. minutum* reached the stationary growth phase after 12 days. Thus, cultures of 6 days age were in early exponential phase, 11 days in late exponential phase, 19 days in stationary phase and of 27 days in late stationary phase. Analyses were performed after 3 h. The same experiment was run with *A. minutum* supernatant added to *C. neogracile*.

Experiment 5—Duration of the Effect of A. minutum Supernatant upon C. neogracile

Supernatant of *A. minutum* (18 days) culture was separated from the cell pellet and kept in the dark at 16°C. After 0, 1, 3, 6 and 9 h, supernatant was added to *C. neogracile* cultures (n=9) and analyses were performed after 1 h of interaction.

Experiment 6—Effects of A. minutum Supernatant upon C. neogracile Photosynthetic Performance

Supernatant of *A. minutum* (17 days) culture filtered on 0.2 µm cellulose acetate (CA) filter (Minisart, Sartorius, Göttingen, Germany) was added to *C. neogracile* cultures in exponential phase. Oxygen concentration was measured with an SDR Oxygen SensorDish */Reader/*® (Presens, Regensburg, Germany) in cultures kept in the dark (respiration only) and in the light (photosynthesis and respiration) from 45 min to several hours of incubation. To measure gross production of O_2 attributable to photosynthesis, consumption of O_2 in cultures kept in the dark was subtracted from values of O_2 in cultures kept in the light.

Experiment 7—Effect of A. minutum Supernatant upon C. neogracile OJIP Curve

Supernatant of *A. minutum* (14 days) culture filtered on CA filter was added to *C. neogracile* cultures in exponential phase. After 15-min of incubation in the dark, Chl *a* fluorescence induction transient (OJIP) curves and FCM measurements were performed on cultures to determine which part of photosynthetic electron transport of *C. neogracile* was affected. Measurements were performed using an AquaPen-C AP-C 100 (Photo Systems Instruments, Czech Republic) applying the internal protocol with blue light (455 nm). A suite of variables, including F_o, F_i, F_j, F_m, S_m, M_o, Ψ_o, RC/ABS, was calculated [42]. These calculations correspond with the initial fluorescence of the culture (F_0), at steps J and I (F_j and F_i), the maximum fluorescence (F_m), the slope of the fluorescence rise, assimilated to the rate of primary quinine electron acceptor (Q_A) reduction (M_o), the multiple-turnover Q_A reduction (S_m), the probability for a electron to go from Q_A to the plastoquinone (PQ) pool (Ψ_o) and the total number of active reaction centers (RC) per unit of absorption (RC/ABS) (Table 1). Four steps can be observed (O, J, I and P), corresponding to different redox states of PSII [1, 42].

Flow cytometry (FCM) measurements

Measurements were conducted with a FACSCalibur (Becton Dickinson) flow cytometer, with an argon blue laser (488 nm) and three fluorescence detectors: FL1 (green, 530 nm), FL2 (orange, 585 nm), and FL3 (red, 670 nm). Red fluorescence is linearly correlated with chlorophyll content of cells and was used to discriminate living microalgal cells. Forward Scatter (FSC, light scattered at less than 10 degrees) and Side Scatter (SSC, light scattered at a 90-degree angle) were also measured. Cultures were stained for 30 min with SYTOX® green (Molecular Probes, Invitrogen, Eugene, Oregon, USA) at a final concentration of 0.05 µM to detect dead cells [32]. SYTOX green staining was first tested and confirmed on *C. neogracile* cultures killed by freezing and formaldehyde fixing (data not shown). All samples were filtered at 80 µm before analyses to avoid clogging the flow cytometer. To allow comparison between days, the same settings of the instruments were used for all experiments. All microalgal cells were detected by FL3 fluorescence and cell densities were estimated from the flow-rate measurement of the flow cytometer [30] as all samples were run for 30 s.

Table 1 List of selected OJIP parameters measured on *C. neogracile* exposed or not to *A. minutum* supernatant

Description		*C. neogracile* without *A. minutum* supernatant		*C. neogracile*+*A. minutum* supernatant		*p*
		Mean	SD	Mean	SD	
F_0	Initial fluorescence, all RC are open	6,174.67	±71.77	3,210.33	±127.24	**
F_j	Fluorescence at step J	11,512.00	±288.11	5,231.00	±148.01	**
F_i	Fluorescence at step I	12,146.67	±155.15	5,609.67	±204.02	**
F_m	Maximal fluorescence, all RC are closed, complete reduction of the photosynthetic electron transport chain	13,801.67	±325.15	6,231.33	±196.19	**
M_o	Approximation of the slope at the origin of fluorescence rise, attributed to the rate of Q_A reduction	1.69	±0.11	1.54	±0.02	NS
S_m	Multiple-turnover Q_A reduction events	433.07	±29.07	519.88	±99.08	NS
$F_v/F_m = (F_m - F_o)/F_m$	PSII efficiency, probability that an absorbed photon will be trapped by the PSII RC with the resultant reduction of Q_A	0.55	±0.01	0.48	±0.01	**
Ψ_o	Probability of electron transfer from Q_A to PQ pool	0.30	±0.01	0.33	±0.02	*
RC/ABS	Total number of active reaction centers per absorption	0.23	±0.01	0.21	±0.00	*

NS nonsignificant

*$p<0.05$, **$p<0.01$, significantly difference values

Differences between analytical replicates are less than 10% (CV=7.7%, n=10).

Statistics

Effects of *A. minutum* cultures or culture fractions upon *C. neogracile* were tested using one-way ANOVAs at each time measurement with the software STATGRAPHICS® Plus 5.1. When variance homogeneity was not confirmed, the non-parametric Kruskall–Wallis test was used. The test of rank used was the Tukey test. Significant differences between *A. minutum* supernatant exposed and non-exposed *C. neogracile* cultures for OJIP parameters were assessed using *t*-tests.

Results

An early observation that informed subsequent experiments was that not all *C. neogracile* cells in a culture responded equally to *A. minutum* exposure. In experiment 2, we observed that FSC and SSC of some *C. neogracile* cells decreased (by 70 to 75% for mean FSC and by 20% for mean SSC compared to control cultures after 24 h) when *A. minutum* cells were added to cultures (Fig. 1a). This decrease in FSC and SSC yielded two distinct populations of *C. neogracile*: (1) unmodified cells, with FSC and SSC similar to control cells and (2) modified cells, with decreased FSC and SSC when exposed to *A. minutum* compared to control cultures (Fig. 1a). Affected *C. neogracile* cells also showed decreased FL3 (red fluorescence) upon exposure to *A. minutum* (Fig. 1b). The percentage of dead *C. neogracile* cells, detected by SYTOX® green staining, did not change significantly in the presence of *A. minutum* in any experiment. Even during the 4 days of experiment 2, no dead cells were detected by SYTOX green staining. Cultures of *C. neogracile* had no effect upon *A. minutum* cells (data not shown). Microscopic observations were also done to confirm FCM results (Fig. 2). Modified cells were smaller, with more-condensed cytoplasm and chlorophyll and reduced chlorophyll fluorescence (Fig. 2).

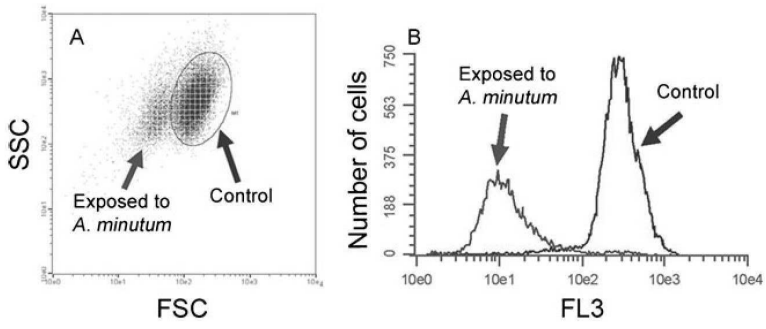

Figure 1 **a** Cytogram of *C. neogracile* cells exposed to *A. minutum* or not (*red circle*) depending on FSC and SSC. **b** Histogram of FL3 (red fluorescence) of *C. neogracile* cells exposed to *A. minutum* or not

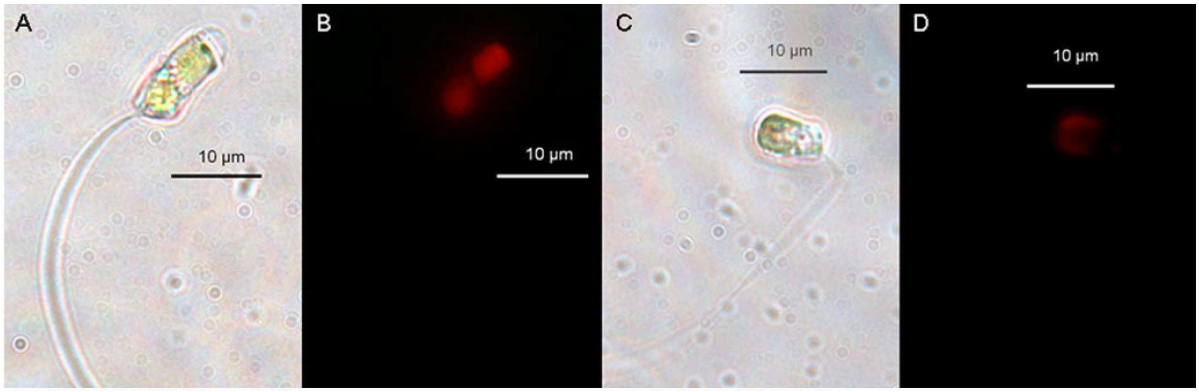

Figure 2 Control cells of *C. neogracile* (**a**, **b**) and cells exposed 2 h to *A. minutum* supernatant (**c**, **d**) under white light (**a**, **c**) and red epi-fluorescence light (**b**, **d**). Scale bar=10 µm

Experiment 1—Effect of *A. minutum* Culture Fractions upon *C. neogracile*

Red fluorescence of *C. neogracile* decreased significantly after 30 min of interaction with *A. minutum* cultures and supernatant ($p<0.01$), and after 2 h of interaction with *A. minutum* cells resuspended in new medium ($p<0.01$). There was no centrifugation effect, as *A. minutum* cells centrifuged and resuspended in the same medium had similar effects as non-centrifuged cells (data not shown). The decrease in *C. neogracile* red fluorescence was significantly sharper with whole *A. minutum* culture than with supernatant (Fig. 3).

Experiment 2—Long-term Effect of *A. minutum* upon *C. neogracile*

Mean cell densities in control cultures of *C. neogracile* increased during the experiment from 450,000 cells ml^{-1} to 830,000 cells ml^{-1}; whereas, cell densities of cultures exposed to *A. minutum* culture or supernatant decreased significantly to less than 50,000 cells ml^{-1} after 96 h of exposure (Fig. 4). Cultures of *C. neogracile* exposed to heated supernatant decreased to less than 300,000 cells ml^{-1} after only 96 h. Both supernatant and heated supernatant induced significant decreases in *C. neogracile* red fluorescence and percentage of unmodified cells for all sampling hours, but the effect was much stronger with non-heated supernatant than with heated supernatant (Fig. 4). Overall, the strongest effect was obtained with the whole culture.

Experiment 3—Effect of *A. minutum* Cell Density upon *C. neogracile*

After 4 h of interaction, a threshold of 200 cells ml^{-1} of *A. minutum* for 200,000 cells ml^{-1} of *C. neogracile* (ratio of 1:1,000) induced a significant decrease in red fluorescence and percentage of unmodified cells of *C. neogracile* ($p<0.05$, Fig. 5). Decreases in *C. neogracile* red fluorescence (Fig. 5) and percentage of unmodified cells were observed with increasing cell density of *A. minutum*.

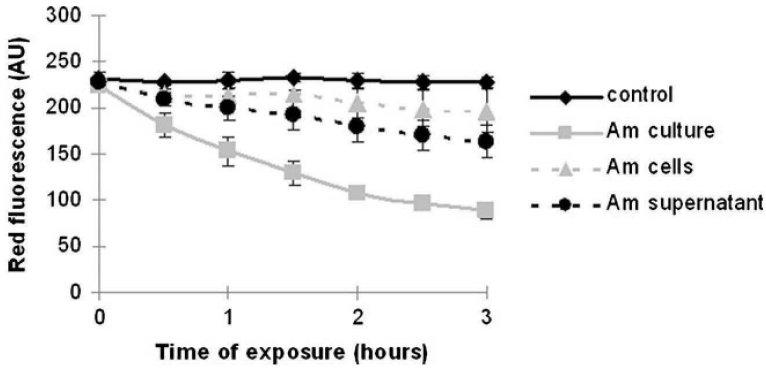

Figure 3 Red fluorescence, in arbitrary units, of *C. neogracile* depending on time of exposure to *A. minutum* (*Am*) cultures, cells or supernatant (mean, standard deviation, $n=3$)

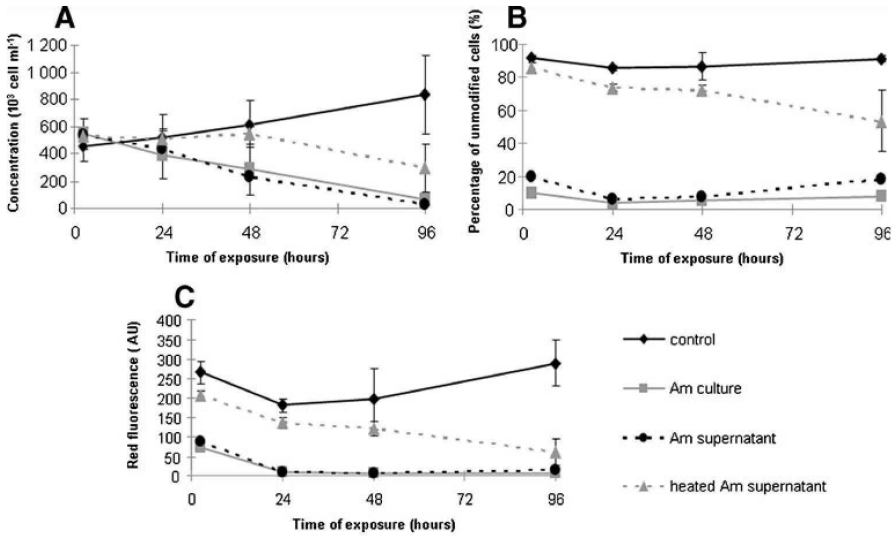

Figure 4 **a** Cell concentration, **b** percentage of unmodified cells and **c** red fluorescence (FL3, in arbitrary units) of *C. neogracile* cultures after 3 to 96 h of exposure to *A. minutum* culture (*Am culture*), supernatant (*Am supernatant*) or supernatant heated during 20 min at 100°C (mean, standard deviation, n=3)

Experiment 4—Effect of *A. minutum* Age upon *C. neogracile*

After 3 h of interaction, culture and supernatant of *A. minutum* caused significant decreases in *C. neogracile* red fluorescence and percentage of unmodified cells (Fig. 6), regardless of the age of the culture (p<0.01). Exposure to *A. minutum* cultures in stationary phase (19 and 27 days) caused stronger effects upon *C. neogracile* than 6 and 11 day-old cultures (Fig. 6a, b). The effect of *A. minutum* supernatant was also significantly enhanced with the age of the filtered culture (Fig. 6c, d). Cultures used for the following experiments were at least 12 days old (early stationary phase).

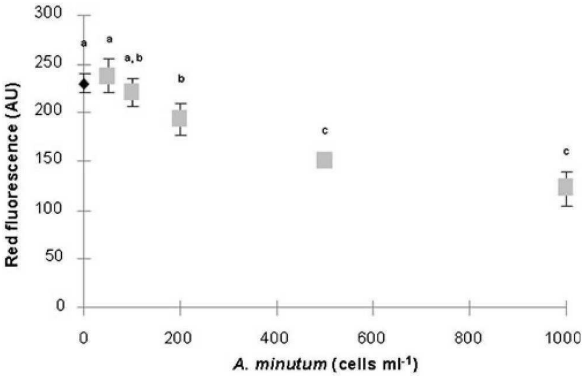

Figure 5 Red fluorescence, in arbitrary units, of *C. neogracile* alone or exposed to *A. minutum* cultures (mean, standard deviation, n=3). *Black lozenge C. neogracile* alone (*control*), *grey squares C. neogracile* exposed to *A. minutum* of different concentration for 4 h. ANOVA groups are represented with p<0.05

Experiment 5—Duration of the Effect of *A. minutum* Supernatant upon *C. neogracile*

Supernatants from *A. minutum* separated from cells for 0 to 6 h induced significant decreases in *C. neogracile* red fluorescence (p<0.01, Fig. 7). After 9 h without cells, the supernatant had no effect upon *C. neogracile* red fluorescence (p>0.05, Fig. 7).

Experiment 6—Effects of *A. minutum* Supernatant upon *C. neogracile* Photosynthetic Variables

Cultures maintained in the dark consumed O_2 attributable to respiration. Significant differences were observed between *C. neogracile* cultures exposed or not to *A. minutum* supernatant, which consumed more O_2 attributable to respiration (p<0.05, Fig. 8a). Cultures maintained in the light produced O_2 through photosynthesis and consumed O_2 through respiration. The mean production of O_2 by photosynthesis was measured by subtracting dark values from light values ([respiration+photosynthesis]−respiration). The mean photosynthetic production of O_2 of *C. neogracile* significantly decreased when exposed to *A. minutum* supernatant (p<0.01, Fig. 8b).

Experiment 7—Effect of *A. minutum* Supernatant upon the *C. neogracile* OJIP Curve

The OJIP curves of *C. neogracile* cultures exposed or not to *A. minutum* supernatant exhibited the same shape, with all OJIP transient phases present (Fig. 9). Nevertheless, all fluorescence values at each phase (F_o, F_j, F_i, F_m) were significantly lower when *C. neogracile* was exposed to *A.*

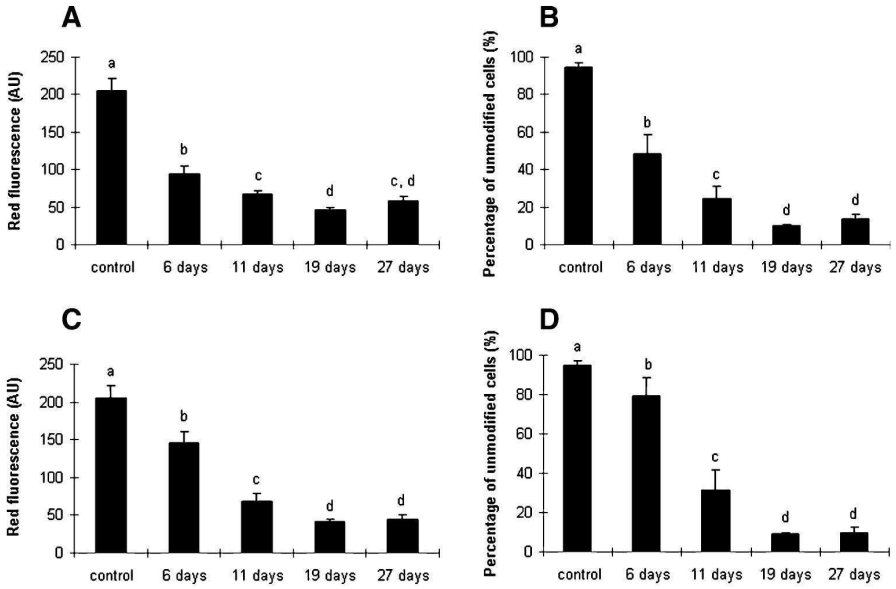

Figure 6 Red fluorescence, in arbitrary units (**a**, **c**) and percentage of unmodified cells (**b**, **d**) of *C. neogracile* after 3 h of exposure to *A. minutum* cultures (**a**, **b**) and supernatant (**c**, **d**) of 6, 11, 19 and 27 days (mean, standard deviation, n=3). ANOVA groups are represented with $p<0.05$

minutum supernatant (Table 1). Quantum yield (QY=F_v/F_m, or photosynthetic efficiency) and total number of active reaction centers per absorption (RC/ABS) also decreased with *A. minutum* supernatant. Probability Ψ_o of electron transfer from the primary quinone electron acceptor in PSII (Q_A) to the plastoquinone (PQ) pool increased in presence of supernatant (Table 1). Multiple-turnover Q_A reduction events (S_m) and the rate of Q_A reduction (M_o) were not affected by the addition of *A. minutum* supernatant ($p>0.05$). FCM FL3 values were also significantly lower when *C. neogracile* was exposed to *A. minutum* supernatant (103.9 fluorescence units for control cultures and 68.1 for supernatant, $p<0.01$).

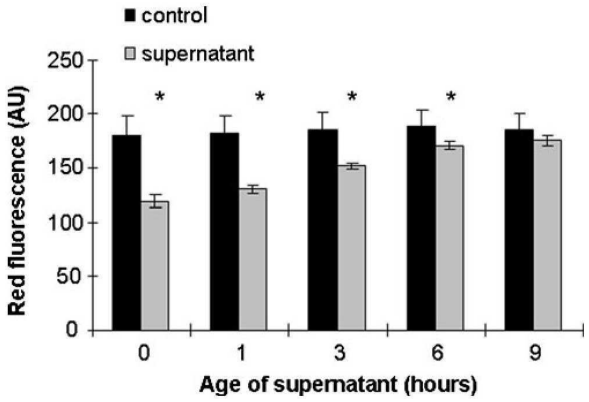

Figure 7 Red fluorescence, in arbitrary units, of *C. neogracile* cultures exposed or not to *A. minutum* supernatant of 0 to 9 h old (mean, standard deviation, n=9). Exposure lasted 1 h. Significant differences are represented by an *asterisk*

Discussion

In this study, usage of both flow-cytometric (FCM) and photosynthetic-performance techniques provided several new insights into allelopathic interactions between *A. minutum* and *C. neogracile*. FCM allowed early detection of slight modifications in fluorescence for numerous samples and conditions within a short time (1 h for 60 samples). In these analyses, we observed that FSC and SSC values of *C. neogracile* decreased for cells exposed to the dinoflagellate *A. minutum*. These quantitative variables are related to morphological changes, such as in shape, cytoplasmic characteristics or cell-surface. These modifications were, however, very difficult to observe and quantify under the microscope (Fig. 2). Morphological changes in targeted cells following allelopathic interactions have been reported previously, but only using microscopic observations [46, 47]. For example, Tillmann et al. [47] described more-elongated cells of *Dunaliella salina* exposed to *Alexandrium ostenfeldii*. Conversely, the dinoflagellate *O. marina* became round when exposed to the haptophyte *P. parvum* [46]. These morphological changes are possibly responsible for observed behavioral modifications and loss of motility [46, 47]. Formation of temporary cysts of *Scrippsiella trochoidea* was also observed when this species was exposed to *Alexandrium tamarense*, *Karenia mikimotoi* and *Chrysochromulina polylepis* [10].

Besides morphological modifications, the dinoflagellate *A. minutum* induced, after only 30 min of interaction, a significant decrease in *C. neogracile* red fluorescence, autofluorescence related to chlorophyll content or function.

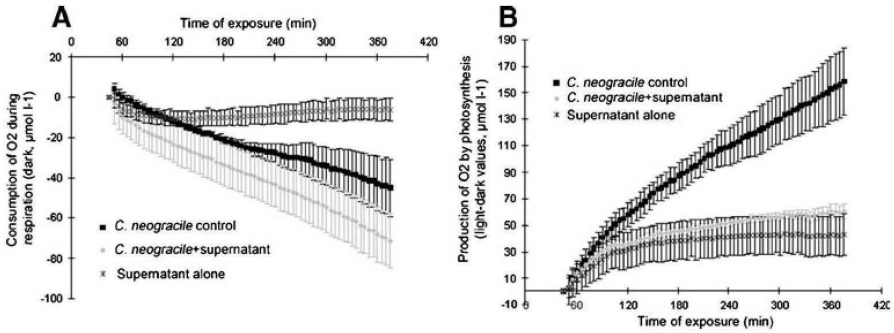

Figure 8 O_2 consumption/production of *C. neogracile* cultures under light conditions (**a**) and during photosynthesis—light–dark values (**b**) for control cultures or cultures exposed to *A. minutum* supernatant. *Black square*s are *C. neogracile* control cultures, *grey lozenges* are *C. neogracile* cultures exposed to *A. minutum* supernatant and *black cross* is *A. minutum* supernatant alone (mean, standard deviation, $n=3$)

In less than 3 h, chlorophyll content is unlikely to change, so the change in FL3 is probably related to altered photosynthetic efficiency (see later in this paragraph). This decrease in red fluorescence was observed for all the cells, regardless of their FSC and SSC values, indicating that the allelochemicals impacted the photosynthetic apparatus of *C. neogracile*. Measurements of photosynthesis were thus performed to analyse in more detail these effects. The decreases in FSC and SSC values may not be directly correlated to the decrease in FL3, as these effects could be attributable to several different allelochemicals.

Effects of *A. minutum* upon *C. neogracile* were not only detectable with Chl *a* fluorescence, but also with photosynthetic production of O_2. Presence of *A. minutum* supernatant decreased *C. neogracile* photosynthetic abilities to produce O_2 to almost no production after only 45 min, which is in quantitative agreement with the decrease in Chl *a* fluorescence observed. Conversely, cells of *C. neogracile* exposed to *A. minutum* supernatant consumed significantly more O_2 from respiration than control cells. Photosynthesis, however, seems to be much more reactive than respiration to allelochemicals produced by *A. minutum*.

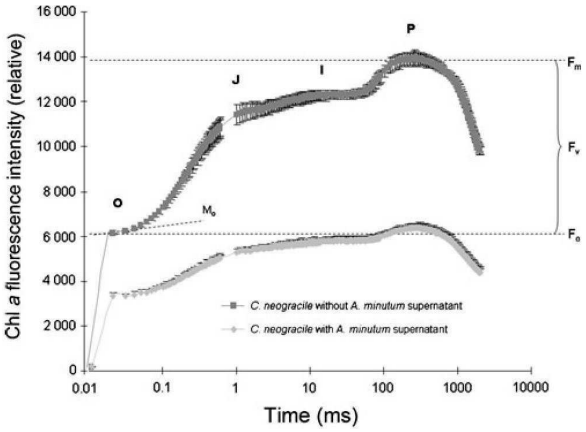

Figure 9 OJIP curve of *C. neogracile* (*Cn*) control (*black*) or exposed for 15 min to *A. minutum* supernatant (*grey*) (mean, standard deviation, $n=3$)

In the presence of *A. minutum* supernatant, the *C. neogracile* OJIP transient curve exhibited the same shape as that of control cultures, but with lower fluorescence values (about half) and the same cell density. The OJIP transient curve is separated into three steps, following the electron transfer through the PSII acceptor side, the PQ pool and beyond the Cytb/f [1]. This curve reflects, among other parameters, the opening and closure of reaction centers (RC) of chlorophyll within the cell. The same decrease in fluorescence values has been shown previously for cells under different kinds of stress, such as N-limitation [2] or addition of chromate [3]. The O, J, I and P steps were still visible in *A. minutum*-exposed *C. neogracile* cells, meaning that electron transfer through the photosynthetic system was still active, and the allelochemicals did not block electron transport through the photosynthetic system. The performance index of PSII is the combination of three independent processes: (1) the total number of active RC per absorption (RC/ABS), (2) the QY, or PSII efficiency ($F_v/F_m=(F_m-F_o)/F_m$), and (3) the efficiency with which an electron can move into the electron transport chain (ψ_0) [3]. RC is a complex of proteins, pigments and other co-factors where the primary energy-conversion reactions of photosynthesis take place; i.e. photosystems II and I and all other intermediates such as plastoquinone (PQ) form one RC unit.

F_o and F_m fluorescences are, respectively, dependent upon the assumption that all RCs are open and closed, respectively [2]. The F_j and F_i fluorescences are attributable to the closure of only some RCs. The ratio F_v/F_m is the probability that an absorbed photon will be trapped by PSII with resultant reduction of Q_A [44]. All these values decreased when *C. neogracile* was exposed to *A. minutum* supernatant, which implies a decrease in total RC number. Indeed, the fewer RCs, the less the cells fluoresce; cells of

C. neogracile exposed to *A. minutum* had lower RC/ABS values, and thus exhibited less-active RCs. As cell number did not change (verified by flow cytometry, data not shown), the decrease in RC number after 15 min of exposure to the supernatant was not caused by a difference in cell number, but rather by a difference within cells. The decrease in *C. neogracile* F_v/F_m in the presence of *A. minutum* indicates decreased photosynthetic efficiency of *C. neogracile*, and the fewer RCs, the lower the probability that a photon will enter PSII, leading to a decrease in F_v/F_m. M_o is an approximation of the slope, at the origin of the rise of fluorescence, attributed to the rate of Q_A reduction [2]. *C. neogracile* M_o did not change under exposure to *A. minutum* supernatant, suggesting that remaining RCs were still active, and Q_A reduction remained possible. This interpretation is also supported by the S_m value, which did not change either, re-enforcing the suggestion that multiple-turnover reduction events of Q_A remained the same. Thus, Q_A was not affected by *A. minutum* allelochemical(s). Ψ_0 increased during allelopathic stress, meaning that, when an electron was caught by Q_A, it entered the electron transport chain more often. Parameters S_m and ψ_0 are proportional to the number of PSII centers able to move electrons from Q_A to PQ pools *via* the secondary quinone electron acceptor in PSII, Q_B [2]. Thus, *C. neogracile* cells were not able to trap as many photons when they were exposed to *A. minutum* supernatant, but when a photon was trapped, the electron transfer was more efficient.

This is the first time *Alexandrium* spp. allelochemicals have been proven to have an effect upon photosynthesis of target cells, and also the first time details of the mechanism of action of these allelochemicals have been investigated. The biochemical mechanisms by which allelochemicals interact with the target cell photosynthetic reaction center, however, remain unknown. Such effects upon photosynthesis have been described for some cyanobacteria (*Fischerella* sp.) against *Monoraphidium* sp. chloroplasts [38]. Von Elert and Jüttner [52] demonstrated inhibition of the PSII-mediated, photosynthetic electron transport in cyanobacteria (*Anabaena* sp.) in the presence of an allelopathic compound produced by another cyanobacteria *Trichormus doliolum*. The same action on PSII-mediated, photosynthetic electron transport (with reduction of F_v/F_m) has been reported for the cyanobacterium *Fischerella* sp., leading to death of the target species, *Chlamydomonas* sp. [13]. Cyanobacteria appear to produce many different allelochemicals that are active against photosynthesis [40]. The allelopathic compound(s) produced by *A. minutum* may have the same characteristics leading to loss of RCs, and thus decreases in red fluorescence in *C. neogracile*. The allelochemicals produced by *A. ostenfeldii* had no effect on photosynthesis of several autotrophic protists [47], probably indicating that different *Alexandrium* species may produce different allelochemicals. Furthermore, different target species may respond in different ways, and *C. neogracile* was not tested by Tillmann et al. [47].

The supernatant of *A. minutum* culture exhibited strong allelopathic activity against *C. neogracile*, as observed previously with different *Alexandrium* species co-incubated with various target phytoplankton species. When culture fractions were tested in the present study, Chl *a* fluorescence decreased faster with whole cultures than with supernatant, which is in agreement with previous studies [36, 45, 46]. Cultures had more allelopathic effects, possibly because cells continuously release allelopathic compounds into the medium. Cells without supernatant—resuspended into a new medium and free of old allelopathic compounds— had to release sufficient quantities of allelopathic compounds to cause inhibitory effects, thus explaining the lag time to obtain a significant effect upon *C. neogracile*. In 2 h, *A. minutum* was able to produce sufficient quantities of allelochemicals to have significant effects upon *C. neogracile*. Thus *A. minutum* cells would be able to quickly outcompete surrounding microalgal cells. Nevertheless, as allelochemicals lose their activity after a few hours, cells need to constantly produce allelochemicals.

Effects of *A. minutum* were dose-dependent and appeared gradually from a cell density of 200 cells ml^{-1}. This dose effect has been observed in many allelopathic species [24, 36, 46, 53, 57] and has been described in the *Alexandrium* genus with different target species [5, 48], including *C. neogracile* [4]. Indeed, more cells release more allelochemicals into the medium, but when there are more target cells, less-intense allelopathic effects are observed. Cell densities used in this study (from 50 to 13,000 cell ml^{-1}) were in agreement with those found in the literature [4, 48] and with natural population data (REPHY). Natural bloom cell density threshold is species-dependent, generally varying between 5,000 and 10,000 cells l^{-1}, depending upon *Alexandrium* species [54], and can reach more than 100,000 cells l^{-1} in France (e.g. in Thau lagoon, 2004, in Penzé, 2005, REPHY). Our results indicated that a minimum threshold of 200,000 cells l^{-1} of *A. minutum* was sufficient to cause allelopathic effects upon *C. neogracile*. This threshold is often reached during a natural bloom (REPHY) and may modify species composition. Moreover, some species present in natural phytoplanktonic communities may be more sensitive to allelochemicals than *C. neogracile* tested here. Thus, allelopathy may not play a major role in the initiation of a bloom, where cell density is low [23], but can become effective once the bloom is established.

Growth phase of *A. minutum* also had an effect upon *C. neogracile* response. Older and denser cultures released more allelopathic compounds into the medium. Rengefors and Legrand [36] also showed that cultures of the freshwater dinoflagellate *Peridinium aciculiferum* were

more toxic in stationary phase. Similar results have been demonstrated here for *A. minutum*, with allelopathic activity increasing with age until stationary phase [4]. It has been shown for terrestrial plants that carbon-based secondary metabolites production increased when plants were limited by nutrients such as nitrogen [41]. Cells of *C. neogracile* were in exponential growth phase, whereas cells of *A. minutum* used for all the experiments (except the variable-age experiment) were in stationary phase, but the types of effects on *C. neogracile* were the same regardless of *A. minutum* growth phase. As *A. minutum* cells showed more allelopathic effects at the end of their growth, allelopathy may function ecologically to maintain the bloom as long as possible, and probably modify the species succession.

As the putative allelopathic compound (or compounds) was active in the media for less than 9 h, live cells of *C. neogracile* were expected to grow the days following the exposure, but instead cell densities continued to decrease during the 4 days of interaction. Cells of *C. neogracile* were never stained with SYTOX green, even after 4 days of exposure, meaning that they did not lose membrane integrity and that the allelopathic compound(s) probably did not have a lytic effect. Lysed cells would have lost a part of their integrity before disappearing; however, no stained cells were detected by FCM or under the microscope. The lack of growth, and even a decrease in *C. neogracile* counts during the 4 days of exposure to *A. minutum*, confirmed that cells were dying. Cells with reduced photosynthetic efficiency did not produce enough energy to grow, and the decrease in autofluorescence of *C. neogracile* cells could also render the cells difficult to detect with the flow cytometer. Thus *C. neogracile* cells first responded to *A. minutum* exposure with a sharp decrease in FL3 fluorescence and in photosynthetic abilities and then a decrease in cell size or complexity. Once the allelochemicals became inactive (9 h) *C. neogracile* did not resume growth because photosynthetic abilities were compromised. This had already been observed with *A. minutum* supernatant slowing down *C. neogracile* growth during 5 days [4].

As mentioned above, *A. minutum* supernatant acted very rapidly (in less than 15 min) upon *C. neogracile* photosynthetic performance and morphology, but filtrate bioactivity declined within 9 h when stored in the dark at 16°C prior to testing on target cells. Hansen [19] reported similar results for *A. tamarense* supernatant, which lost allelopathic activity within 30 min. No differences between storage in plastic or glass tubes on allelopathic activity were observed (data not shown), suggesting that disappearance of allelopathic activity in 9 h was not attributable to surface binding of allelochemicals. Indeed, Ma et al. [28] showed that allelochemicals can bind to plastic, but not glass tubes. Thus, evaluating responses of *C. neogracile* led us to conclude that *A. minutum* supernatant had “short-term” allelopathic activity (effects within the first minutes of exposure). Furthermore, supernatant of *A. minutum* lost most “short-term” toxicity after 20 min of heating at 100°C, suggesting that the allelopathic compounds involved in this activity were thermolabile. This disappearance of allelopathic effects in 9 h was not associated with bacterial degradation as no difference was observed in loss of allelopathic activity between supernatants prepared from cultures with or without antibiotics (data not shown). Ma et al. [28] also reported that there was no bacterial degradation of *Alexandrium* exudates. Nevertheless, Ma et al. [28] had an allelochemical with a longer "life", which support the hypothesis that different species of *Alexandrium* produce different allelochemicals. As shown in Fig. 4, even if the allelochemicals became inactive in less than 9 h, effects remained on previously exposed *C. neogracile* cells and growth, with an increasing percentage of modified cells after 24 h and a decrease in cell density. It suggests a “long-term” or "residual" effect of allelopathic compounds produced by *A. minutum*, with cells impacted for a long time in the absence of allelochemicals. These “long-term” affected cells just disappeared from the culture as measured by flow cytometer, thus we can assume that they died and were degraded by bacteria present in the cultures. Moreover, this “long-term” effect was only partially reduced after heating *A. minutum* medium at 100°C for 20 min. Similar results have been found on *A. tamarense* and *A. taylori* allelochemicals, which are still partially active after 15 min at 95°C [28] and after 30 min at 100°C [9], respectively. Overall, it cannot be excluded that *A. minutum* produces at least 2 allelopathic compounds (with short-and long-term effects). Similarly, *A. ostenfeldii* supernatant retained allelopathic properties for up to 3 days [47].

Differences in allelochemical characteristics described in the literature are known between species, but also between clones of the same species. Some clones of *A. tamarense* are not demonstrably allelopathic [49] and, among the allelopathic strains, different compounds have been described for this species [4, 9, 28, 55], such as a protein-like compound of more than 10 kDa [9] or a polysaccharide-based compound [55]. Culture media may also influence the chemical characteristics and activity of allelochemicals. Indeed, Ma et al. [28] used K medium with selenium, but Fistarol et al. [11] used f/2 media, and Emura et al. [9] used ESM media. We used L1 medium to grow *A. minutum*. A difference in ionic forces of the media (probably due to differences in trace metal composition) might explain differences in stability of allelochemicals, as well as differences in polarity of the allelochemicals. Finally, these species likely produced several allelopathic compounds that may act synergistically or independently. Thus, the nature of *A. minutum* allelopathic compounds still remains mysterious.

Effects upon target species are quite well studied; nevertheless, the identification and biochemical characterization of the allelochemicals, as well as the mechanism of action of those compounds, remain unclear and still need to be investigated.

This study used for the first time two different methods to assess allelopathic effects of *A. minutum* on *C. neogracile*. Flow cytometry allowed fast and reproducible measurements of these allelopathic effects on *C. neogracile* morphology and physiology, decrease in cell autofluorescence with modifications of cell shape, but without any lysis detected. For the first time, mechanisms of action of allelochemicals of *Alexandrium* were also assessed using photosynthetic-performance analysis. Results demonstrated that *Alexandrium* allelochemicals decreased the RC number of each target cell without affecting the efficiency of electron transfer through these RCs. These effects were only previously demonstrated for cyanobacterial allelochemicals and are described here for the first time with dinoflagellate allelochemicals. Further experiments need to be performed to assess whether this mechanism of action is the same regardless of the target cell.

Acknowledgements The authors would like to thank Isabelle Queau for providing the *C. neogracile* strain and Gary Wikfors for his constructive comments and English corrections. This study was supported by the French "Ministère de l'Education Nationale, de la Recherche et de la Technologie" (MENRT grant). The authors also would like to thank the anonymous reviewers for their help in improving this manuscript.

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VALORISATION SCIENTIFIQUE

✓ Publications

Lelong A., Hégaret H., Soudant P. 2011. Cell-based measurements to assess physiological status of *Pseudo-nitzschia multiseries*, a toxic diatom. *Research in Microbiology* 162: 969-981.

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✓ Abstract

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✓ Communications orales

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